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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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Bozeman, Montana
June, 1955

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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.

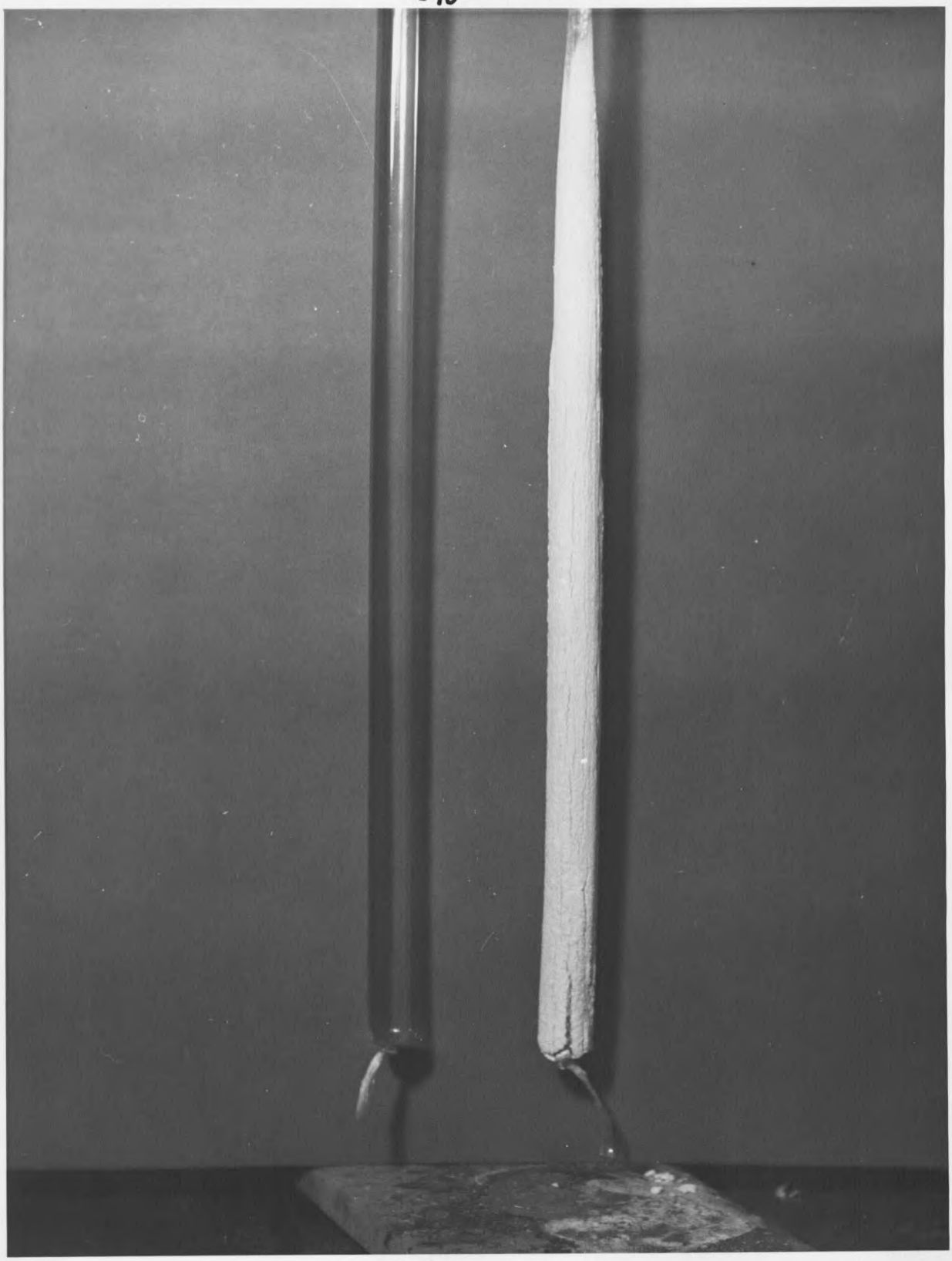


Figure 1

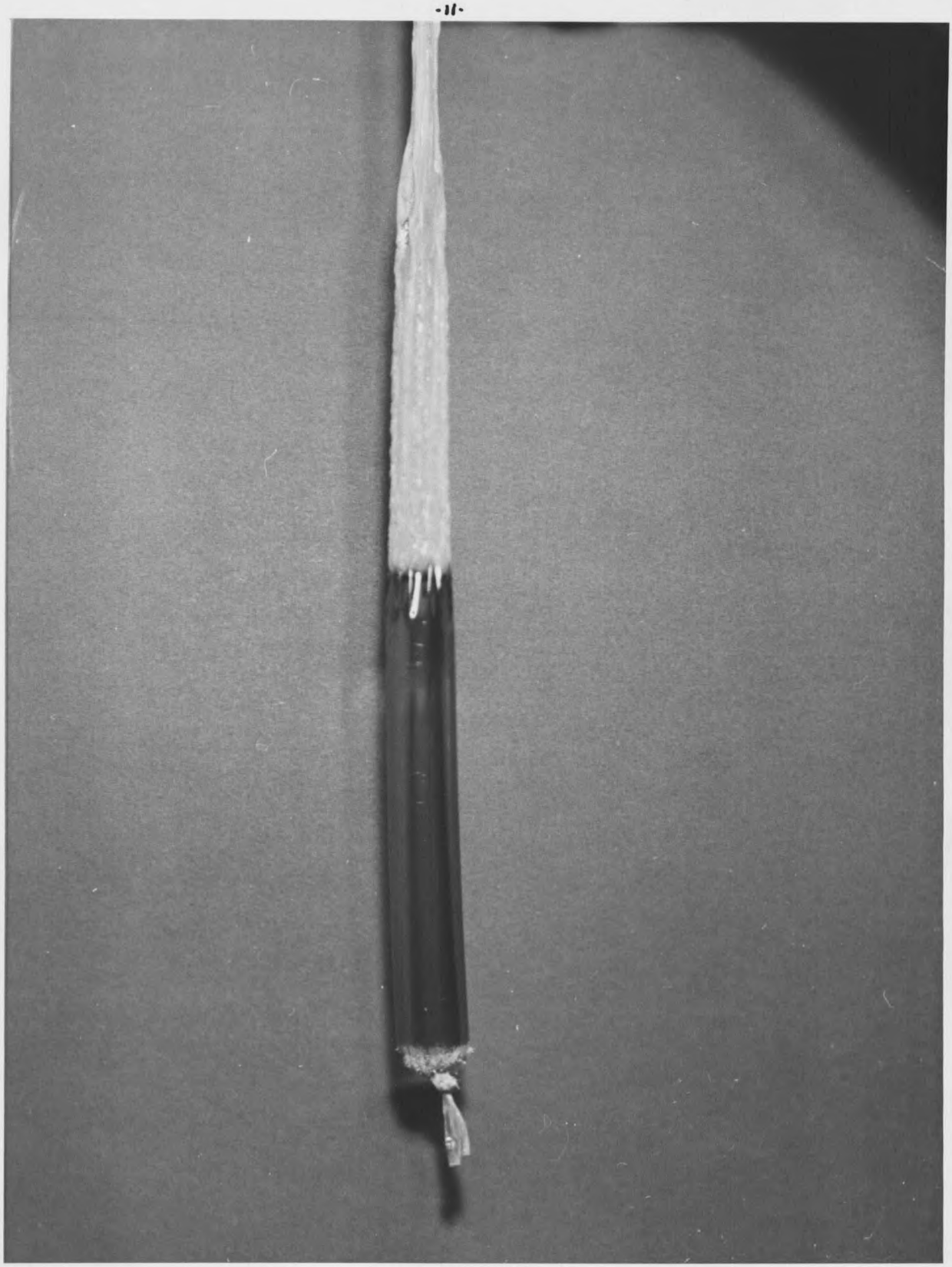


Figure 2

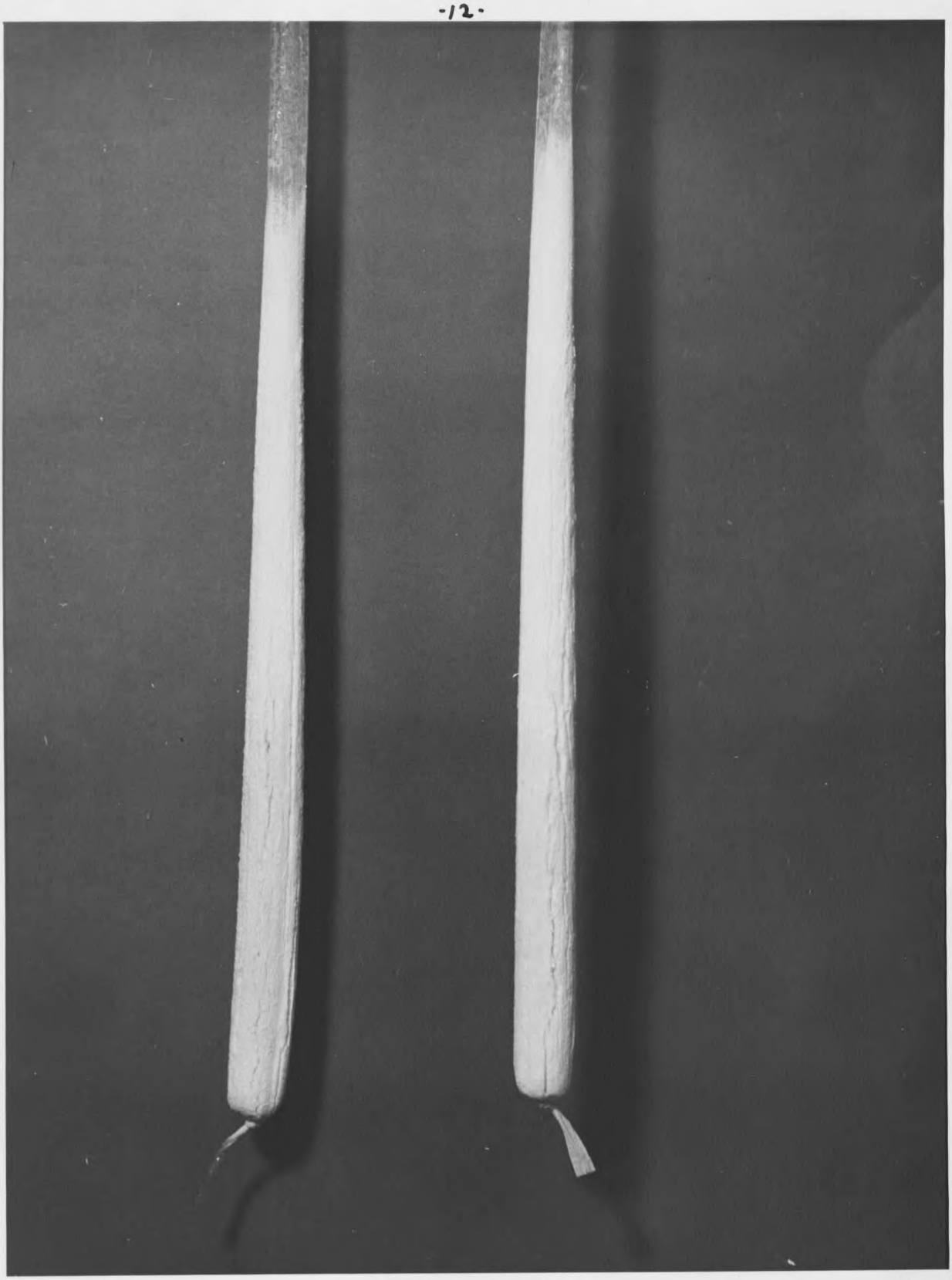


Figure 3

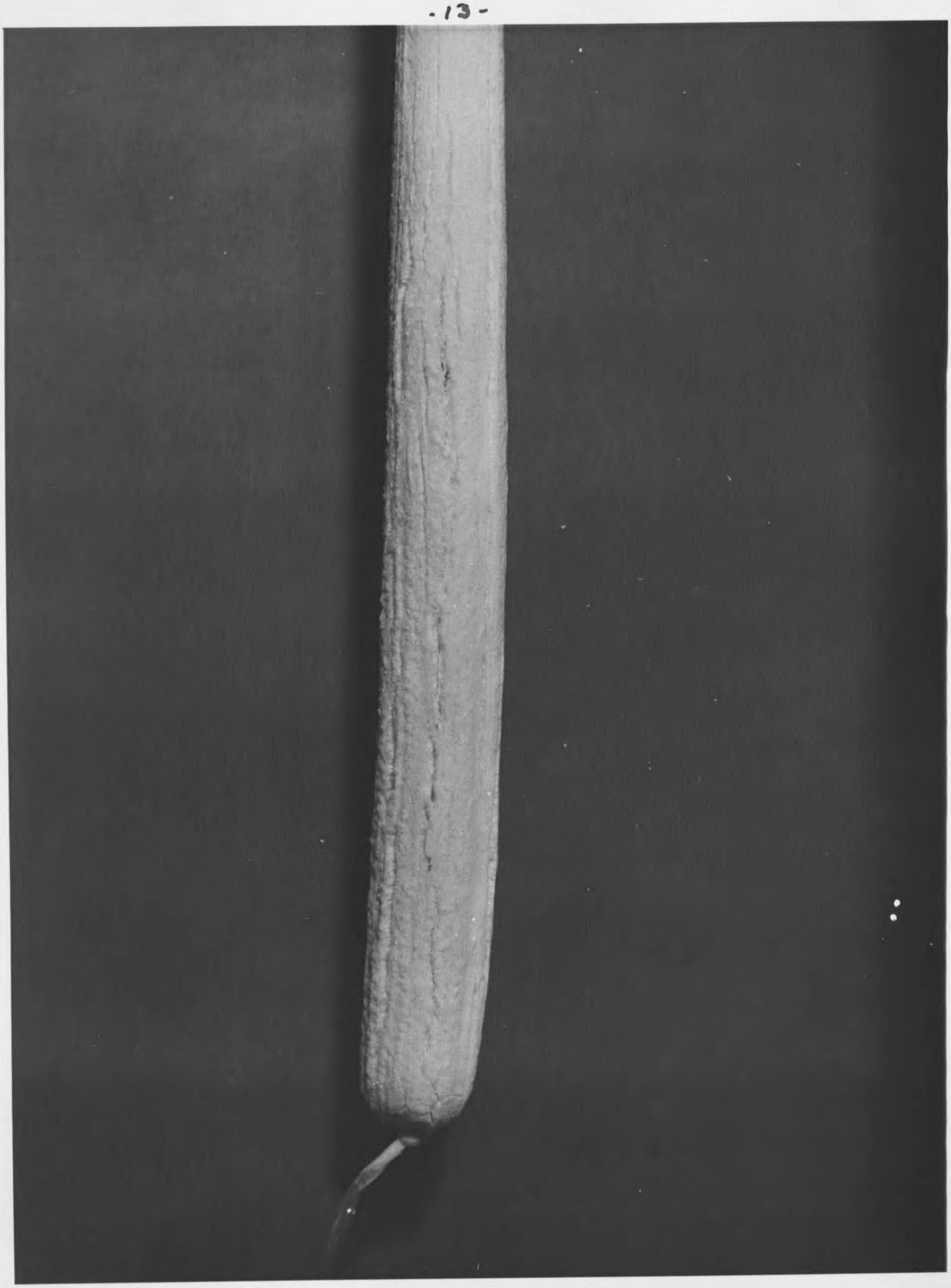


figure 4

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.

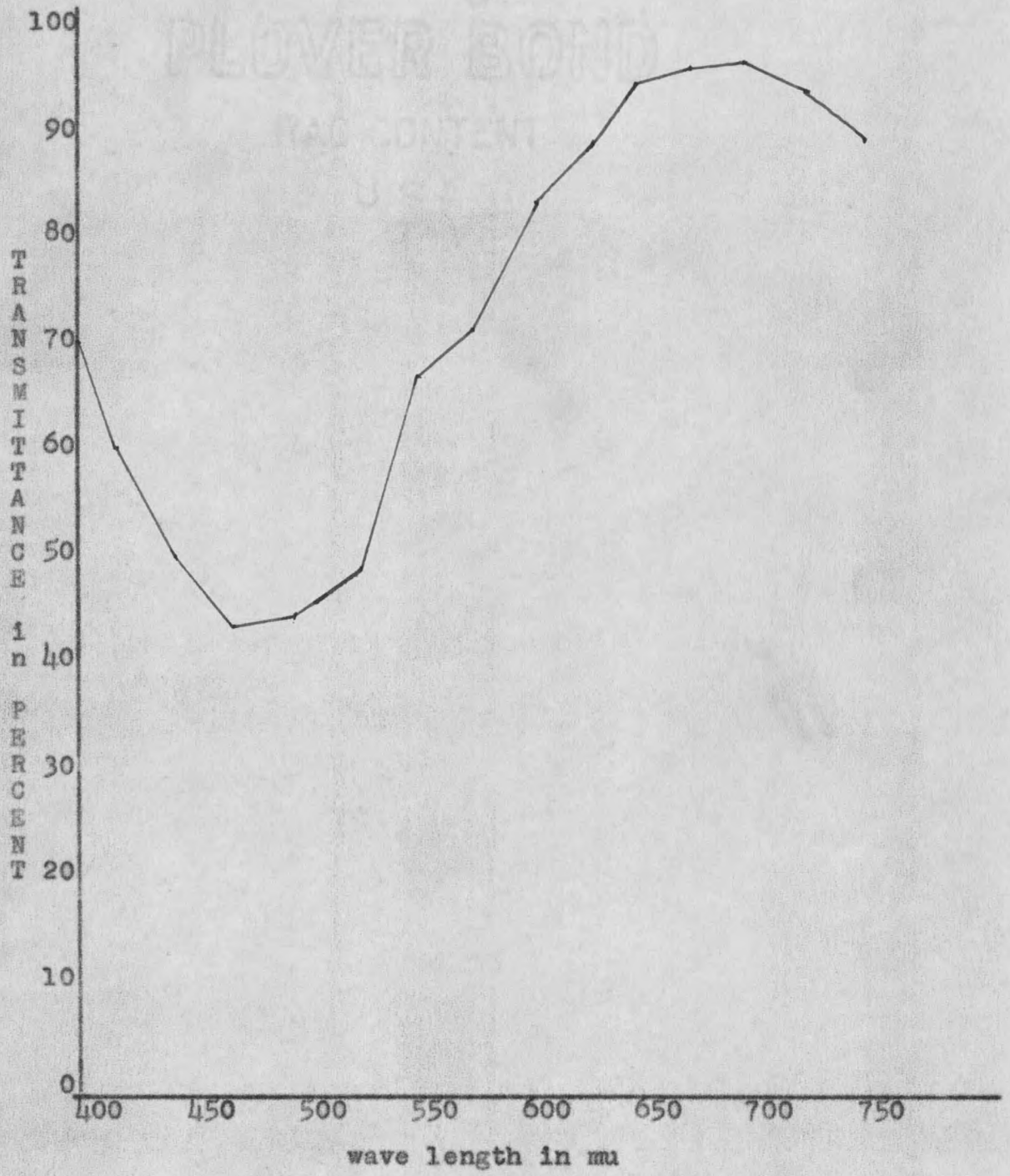
Standard Concentration Curves for Cystine and Tyrosine

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.

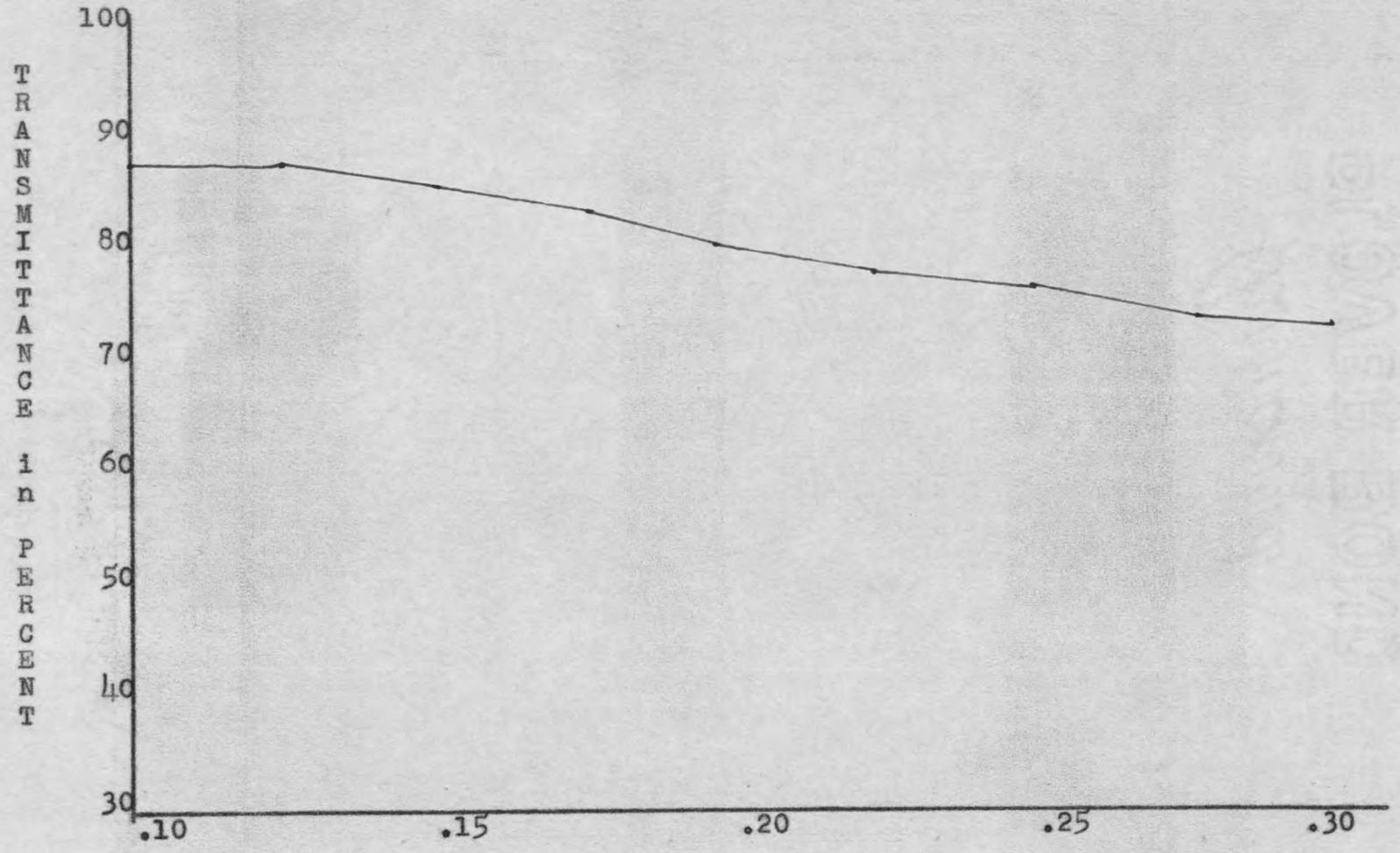
The most sensitive range for cystine was found to be from .10 to .30 mg. per ml. A standard curve with concentrations within this range, and with increments of .025 mg. per ml. was prepared. Figure 6 is the standard cystine curve.

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.



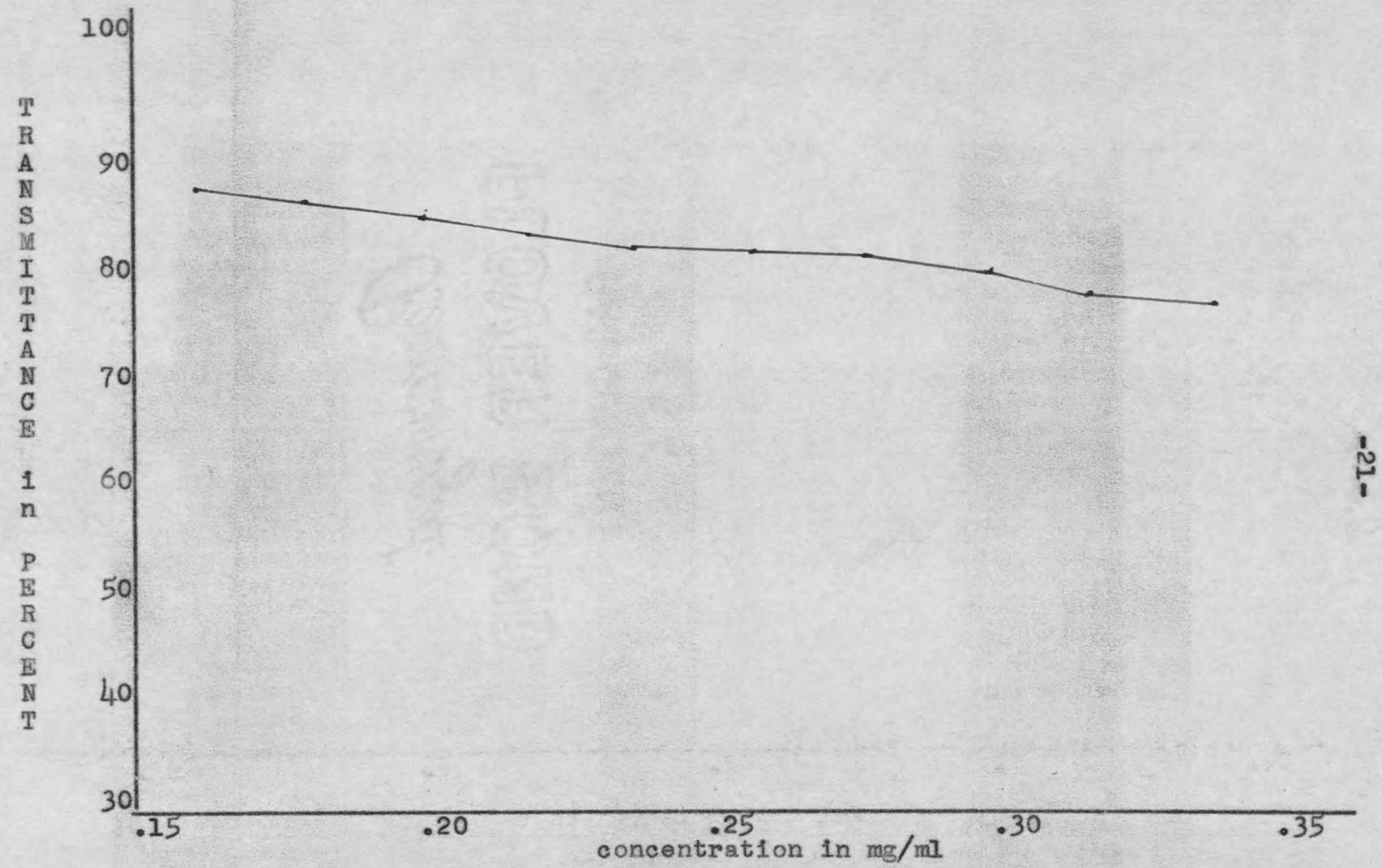
WAVE LENGTH AT MINIMUM TRANSMITTANCE FOR THE TYROSINE TEST

figure 5



CYSTINE STANDARD CURVE at 580 mu

figure 6



TYROSINE STANDARD CURVE at 475 mu

figure 7

Quantitative Analysis of the Encrustations and Protein Hydrolysate Solutions for Cystine and Tyrosine

In order to find sample sizes containing concentrations of cystine and tyrosine within the concentration range of the cystine and tyrosine standards, it was necessary to make numerous trial runs. It was found that one gram of solid outside material and .1 ml. of inside solution contained concentrations of cystine within the range on the standard curve. The same quantities were found to be suitable for the tyrosine test.

Seven bags containing the protein hydrolysate with $(NH_4)_2SO_4$, seven containing NH_4Cl, and four containing NaCl were analysed. The total cystine and tyrosine present in 200 ml. of each of the above solutions were calculated by analysing .1 ml. samples of each solution. Three trials were run in each case and the average value was taken. Table I shows the concentration of cystine and tyrosine in the $(NH_4)_2SO_4$ solution, Table II in the NH_4Cl solution, and Table III in the NaCl solution.

The encrustation on each bag was collected and weighed. One gram samples from each bag were analysed for cystine and tyrosine. The total quantities of cystine and tyrosine in the surface encrustation were then calculated. The percent of cystine and tyrosine passing through the membrane was then calculated.

Table IA

Concentration of Tyrosine in a Hydrolysate Containing $(NH_4)_2SO_4$

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Tyrosine
82.5	.222 mg.	2.22 mg.	200 ml.	444 mg.
82	.226	2.26	200	452
81	.244	2.44	200	488

average total conc. of tyrosine in 200 ml. of solution 461 mg.

Table IB

Concentration of Cystine in a Hydrolysate Containing $(NH_4)_2SO_4$

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Cystine
74	.270 mg.	2.70 mg.	200 ml.	540 mg.
73	.284	2.84	200	568
75.5	.258	2.58	200	516

average total conc. of cystine in 200 ml. of solution 541 mg.

Table IIA

Concentration of Tyrosine in a Hydrolysate Containing NH_4Cl

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Tyrosine
83	.217 mg.	2.17 mg.	200 ml.	434 mg.
82.5	.218	2.18	200	436
83	.217	2.17	200	434

average total conc. of tyrosine in 200 ml. of solution 435 mg.

Table II B

Concentration of Cystine in a Hydrolysate Containing NH_4Cl

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Cystine
75	.260 mg.	2.60 mg.	200 ml.	520 mg.
75	.260	2.60	200	520
75.5	.258	2.58	200	516

average total conc. of cystine in 200 ml. of solution 518.7 mg.

Table IIIA

Concentration of Tyrosine in a Hydrolysate Containing NaCl

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Tyrosine
81	.246 mg.	2.46 mg.	200 ml.	492 mg.
81.5	.240	2.40	200	480
81.5	.240	2.40	200	480

average total conc. of tyrosine in 200 ml. of solution 484 mg.

Table IIIB

Concentration of Cystine in a Hydrolysate Containing NaCl

Reading	Conc. per sample (.1 ml.)	Conc. per ml.	Total Volume	Total Cystine
73	.286 mg.	2.86 mg.	200 ml.	572 mg.
72.5	.289	2.89	200	578
73.5	.280	2.80	200	560

average total conc. of cystine in 200 ml. of solution 570 mg.

RESULTS

Tables IV A and IV B show the percentage of tyrosine and cystine passing from the $(NH_4)_2SO_4$ solution. Notice that a greater percentage of tyrosine passes than cystine. Tables V A and V B and Tables VI A and VI B, showing the percent of tyrosine and cystine passing from the NH_4Cl and NaCl solutions respectively, show the same relationship between the amount of tyrosine and cystine passing. The percentage of the two acids passing from the NH_4Cl solution is only slightly larger than that passing from the NaCl solution, but the percentages from both of these solutions are considerably smaller than the percentages passing from the $(NH_4)_2SO_4$ solution. Note the relationship between the quantity of encrustation and the percentage of cystine and tyrosine passing. The average weight of the $(NH_4)_2SO_4$ encrustation was 55.41 grams and the percent of tyrosine passing was 2.22, while the cystine passing was 2.08%. The average encrustation from the NH_4Cl solution weighed 45.45 grams, with 1.76% tyrosine and 1.66% cystine passing. The NaCl solution had an average encrustation of 42.5 grams, with 1.73% tyrosine and 1.58% cystine passing.

The concentration of cystine and tyrosine per gram of encrustation did not follow the same order, however. The concentration of tyrosine per gram of encrustation was highest in NaCl, next highest in $(NH_4)_2SO_4$, and the least in NH_4Cl. Sodium Chloride contained the highest concentration of cystine

per gram, next highest in $(NH_4)_2SO_4$, and NH_4Cl contained the least.

TABLE IV A

Percent Tyrosine Passing from the Hydrolysate Containing $(NH_4)_2SO_4$

Weight of Surface Crust	Reading	Conc. of Tyrosine per gram	Total Tyrosine Passing	Fraction	% Passing
52.75 g.	83	.210 mg.	11.064 mg.	$\frac{11.064}{461.00}$*	2.40
51.50	85	.181	9.312	$\frac{9.312}{461}$	2.02
57.85	86	.169	9.773	$\frac{9.773}{461}$	2.12
55.84	84	.198	11.064	$\frac{11.064}{461}$	2.40
52.55	84.5	.194	10.188	$\frac{10.188}{461}$	2.21
58.30	85.5	.176	10.261	$\frac{10.261}{461}$	2.23
59.10	86	.169	9.988	$\frac{9.988}{461}$	2.17
55.41 g.		.185 mg.	10.236 mg.		2.22 %

Moles of $(NH_4)_2SO_4$ passing $\frac{55.41}{132} = .420$

Ionic concentration $= .840$ gram cation

Concentration of tyrosine per gram cation of $(NH_4)_2SO_4$ $\frac{10.236}{.840} = 12.18$ mg.

* mg. of tyrosine in 200 ml. of hydrolysate

TABLE IV B

Percent Cystine Passing from the Hydrolysate Containing $(NH_4)_2SO_4$

Weight of Surface Crust	Reading	Conc. of Cystine per gram	Total Cystine Passing	Fraction	% Passing
52.75 g.	80.5	.192 mg.	10.13 mg.	$\frac{10.13}{541}$ *	1.87
51.50	80	.200	10.30	$\frac{10.30}{541}$	1.94
57.85	79.5	.204	11.80	$\frac{11.80}{541}$	2.18
55.84	79	.210	11.73	$\frac{11.73}{541}$	2.17
52.55	79.5	.204	10.72	$\frac{10.72}{541}$	1.98
58.30	79	.210	12.24	$\frac{12.24}{541}$	2.26
59.10	80	.200	11.82	$\frac{11.82}{541}$	2.18
55.41 g.		.203 mg.	11.25 mg.		2.08 %

Moles of $(NH_4)_2SO_4$ passing $\frac{55.41}{132} = .420$

Ionic concentration $= .840$ gram cation

Concentration of cystine per gram cation of $(NH_4)_2SO_4$ $\frac{11.25}{.840} = 13.49$ mg.

* mg. of cystine in 200 ml. of hydrolysate

TABLE V A

Percent Tyrosine Passing from the Hydrolysate Containing NH_4Cl

Weight of Surface Crust	Reading	Conc. of Tyrosine per gram	Total Tyrosine Passing	Fraction	% Passing
47.55	85.5	.174 mg.	8.265 mg.	$\frac{8.265}{435}$ *	1.90
44.50	87	.160	6.960	$\frac{6.96}{435}$	1.60
44.30	87	.160	7.134	$\frac{7.134}{435}$	1.64
47.00	85.5	.174	8.178	$\frac{8.178}{435}$	1.90
43.80	85	.183	8.004	$\frac{8.004}{435}$	1.84
46.30	85.5	.174	8.091	$\frac{8.091}{435}$	1.86
44.70	87	.160	7.004	$\frac{7.004}{435}$	1.61
45.45 g.		.169 mg.	7.662 mg.		1.76 %

Moles of NH_4Cl passing $\frac{45.45}{53.50} = .849$

Ionic concentration $= .849$ gram cation

Concentration of tyrosine per gram cation of NH_4Cl $\frac{7.662}{.849} = 9.02$ mg.

* mg. of tyrosine in 200 ml. of hydrolysate

TABLE V B

Percent Cystine Passing from the Hydrolysate Containing NH_4Cl

Weight of Surface Crust	Reading	Conc. of Cystine per gram	Total Cystine Passing	Fraction	% Passing
47.55 g.	80.5	.193 mg.	9.181 mg.	$\frac{9.181}{518.7}$*	1.77
44.50	81.5	.182	8.095	$\frac{8.095}{518.7}$	1.58
44.30	81.5	.182	8.063	$\frac{8.063}{518.7}$	1.55
47.00	80	.200	9.440	$\frac{9.440}{518.7}$	1.82
43.80	81	.184	8.040	$\frac{8.040}{518.7}$	1.55
46.30	80.5	.193	8.922	$\frac{8.922}{518.7}$	1.72
44.70	80.5	.193	8.610	$\frac{8.610}{518.7}$	1.66
45.45 g.		.189 mg.	8.621 mg.		1.66 %

Moles of NH_4Cl passing $\frac{45.45}{53.50} = .849$

Ionic concentration $= .849$ gram cation

Concentration of cystine per gram cation of NH_4Cl $\frac{8.621}{.849} = 10.15$ mg.

* mg. of cystine in 200 ml. of hydrolysate

TABLE VI A

Percent Tyrosine Passing from the Hydrolysate Containing NaCl

Weight of Surface Crust	Reading	Conc. of Tyrosine per gram	Total Tyrosine Passing	Fraction	% Passing
43.4 g.	84	.204 mg.	8.854 mg.	$\frac{8.854}{484}$ *	1.83
41.2	85	.188	7.746	$\frac{7.746}{484}$	1.60
44.8	84.5	.192	8.602	$\frac{8.602}{484}$	1.78
40.7	84	.204	8.303	$\frac{8.303}{484}$	1.71
42.5 g.		.197 mg.	8.376 mg.		1.73 %

Moles of NaCl passing $\frac{42.5}{58.5} = .726$

Ionic concentration = .726 gram cation

Concentration of tyrosine per gram cation of NaCl $\frac{8.376}{.726} = 11.54$ mg.

* mg. of tyrosine in 200 ml. of hydrolysate

TABLE VI B

Percent of Cystine Passing from the Hydrolysate Containing NaCl

Weight of Surface Crust	Reading	Conc. of Cystine per gram	Total Cystine Passing	Fraction	% Passing
43.4 g.	79	.210 mg.	9.114 mg.	$\frac{9.114}{570}$ *	1.60
41.2	78.5	.218	8.893	$\frac{8.893}{570}$	1.57
44.8	78	.222	9.946	$\frac{9.946}{570}$	1.74
40.7	80	.200	8.140	$\frac{8.140}{570}$	1.43
42.5 g.		.212 mg.	9.013 mg.		1.58 %

Moles of NaCl passing $\frac{42.5}{58.5} = .726$

Ionic concentration = .726 gram cation

Concentration of cystine per gram cation of NaCl $\frac{9.013}{.726} = 12.41$ mg.

* mg. of cystine in 200 ml. of hydrolysate

EXPERIMENTAL PART II

A number of control experiments were run using several different amino acids, amino acid salts, and inorganic salts in an effort to find some of the factors governing percrystallization.

A solution containing one gram of alanine in 50 ml. of water was placed in a cellophane casing. A second such solution also containing $(NH_4)_2SO_4$ was prepared. A solution containing one gram of lysine-hydrochloride in 50 ml. of water was placed in a cellophane bag. Cystine-hydrochloride was prepared by dissolving cystine in the minimum amount of 6N HCl which would dissolve all of the cystine and diluting it with water to 50 ml. This solution was placed in a cellophane casing. The sodium salt of cystine was prepared by adding the minimum amount of 6N NaOH which would dissolve a quantity of cystine. The solution was diluted with water to 50 ml. and placed in a cellophane casing. All of these casings were hung up for one week at room temperature.

Results

The volume in each casing was reduced to between 5 and 10 ml. after one week. The solution containing pure alanine showed no percrystallization. The casing containing alanine and $(NH_4)_2SO_4$ had developed an encrustation on the surface. This material gave a positive ninhydrin test, indicating the presence of an amino acid. No encrustation developed on the

casings containing lysine-hydrochloride, cystine-hydrochloride, or the sodium salt of cystine. In the latter two, the casings had leaked due to the weakening of the membrane by acid in the one and base in the other.

Cystine in the Presence of Inorganic Salts

Four solutions containing .5 grams cystine in 50 ml. of water were prepared by dissolving the cystine in minimum quantities of 6N HCl and then diluting with water. To one solution was added one gram of $(NH_4)_2SO_4$, to another one gram of NH_4Cl, to another one gram of NaCl, to the fourth was added two grams of $(NH_4)_2SO_4$. A fifth solution containing one gram of cystine in 50 ml. of water was prepared and to this was added one gram of $(NH_4)_2SO_4$. These solutions were placed in cellophane casings and left hanging for three days.

All the casings had encrustations on the surface, but several casings had leaked. When the encrustation on the other casings were removed, they also broke. The acidity of the solutions were such that they weakened the membranes to the point that they leaked or broke easily. Thus no quantitative results were possible. The encrustations collected, however, produced positive ninhydrin tests. Another attempt using less acid to dissolve the cystine resulted in the same difficulty.

Experiments With Collodion Membranes

Five collodion membranes in the shape of ten inch test

tubes were made and filled with solutions containing alanine, cystine-hydrochloride, sodium salt of cystine, a protein hydrolysate containing $(NH_4)_2SO_4$, and a solution containing cystine, tyrosine, and $(NH_4)_2SO_4$, respectively. These bags were hung up and left for one week at room temperature. At the end of that period, no encrustation had developed on any of the bags, and the volume had been reduced very little. Large beakers were placed around the bags and heat applied with bunsen burners. With heat applied for several hours, and the temperature of the air around the bags as high as 95°C, no percrystallization took place.

Measurement of the Amounts of Inorganic Salts Which Pass Through a Cellophane Membrane

It is necessary to know the relative amount of an inorganic salt which passes through a membrane, in order to judge the effect that salt has on the amount of cystine or tyrosine which will pass through the membrane. Two molar solutions of NH_4Cl, NaCl, $(NH_4)_2SO_4$ and a one molar solution of $(NH_4)_2SO_4$ were prepared. Three 100 ml. quantities of each solution were placed in cellophane casings. The amount of encrustation on each casing was weighed and the percent passing through was calculated. Table VII shows the results. The solubility in grams per 100 grams of water of each salt is also indicated.

TABLE VII

Percent of Inorganic Salt Passing Through a Cellophane Membrane

Salt	Molarity	Conc. in g/100 ml. of H_2O	Sol. in g/100g of H_2O at 20°C	Grams Passed	% Passed
NH_4Cl	2 molar	10.7 g.	37.2 g.	1.500g.	14.01
"	"	"	"	1.366	12.67
"	"	"	"	1.438	13.44
					13.37%
moles passed = .080		Gram cations passed = .080			
NaCl	2 molar	11.7 g.	36.0 g.	1.476g.	12.62
"	"	"	"	1.526	13.04
"	"	"	"	1.500	12.80
					12.82%
moles passed = .077		gram cations passed = .077			
$(NH_4)_2SO_4$	2 molar	26.4 g.	75.4 g.	5.739g.	21.74
"	"	"	"	5.932	22.47
"	"	"	"	6.022	22.81
					22.34%
moles passed = .045		gram cations passed = .090			
$(NH_4)_2SO_4$	1 molar	13.2 g.	75.4 g.	1.581g.	11.98
"	"	"	"	1.620	12.27
"	"	"	"	1.598	12.11
					12.12%
moles passed = .037		gram cations passed = .074			

DISCUSSION

Amino acids and inorganic salts alike would not pass through a collodion membrane under the conditions described in the experimental work. It is conceivable that if it had been possible to make thinner, more delicate collodion membranes, that inorganic salts, and possibly amino acids along with the salts, would have passed through the membranes. However, one might conclude that Kober (7) worked under different conditions or used collodion membranes with additional components when he succeeded in percrystallizing histidine from an amino acid solution.

The fact that neither amino acids in salt free solution, nor amino acid salts would pass through a cellophane membrane, leads to the conclusion that when small quantities of amino acids pass through a membrane along with inorganic salts, they are merely carried along in some way by the salt.

Tables IV, V, and VI show that the $(NH_4)_2SO_4$ solution passed a greater percentage of cystine and tyrosine through a cellophane membrane than either NH_4Cl or NaCl. The $(NH_4)_2SO_4$ encrustation weighed more than the other two salts, which were nearly equal in weight. The quantity of cystine and tyrosine in the solution is constant, thus the greater the amount of salt passing, the greater the total cystine and tyrosine passing. This explains why $(NH_4)_2SO_4$ passed the greatest quantity of cystine and tyrosine through the membrane.

The NaCl encrustation contained the highest concentration of tyrosine and cystine per gram of encrustation followed by $(NH_4)_2SO_4$ and NH_4Cl respectively. When the molar concentrations are calculated, it is found that the molar concentration of $(NH_4)_2SO_4$ is roughly one-half that of the other two salts passing through the cellophane membrane. Ammonium sulfate has twice the cation concentration of NH_4Cl and NaCl, however. Thus doubling the molar concentration of $(NH_4)_2SO_4$, a cation concentration of .840 is obtained, while the cation concentration of NH_4Cl remains at .849 and the cation concentration of NaCl remains at .726.

One would expect $(NH_4)_2SO_4$ and NH_4Cl to promote the passage of higher concentrations of cystine and tyrosine through the cellophane membrane than NaCl, if a correlation existed between this phenomenon and ionic concentration. We find, however, that $(NH_4)_2SO_4$ contains the highest concentration of tyrosine and cystine per gram cation followed by NaCl and NH_4Cl respectively. Thus no correlation seems to exist between concentration of cations passing through the membrane and the concentration of cystine and tyrosine passing through the membrane with the inorganic salt.

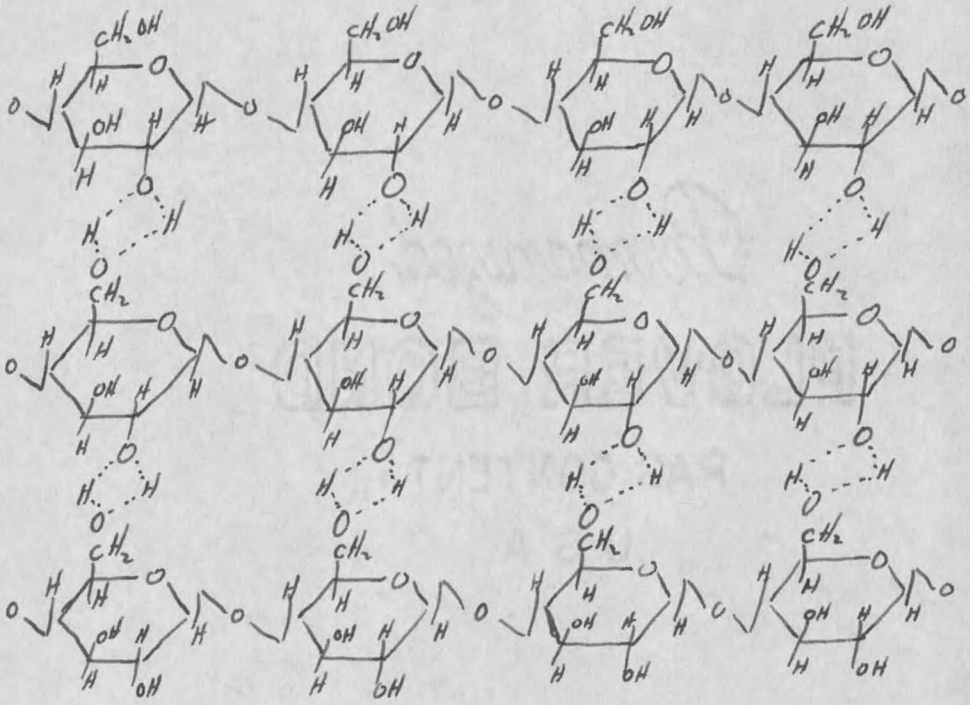
Table VII indicates, as do the results in Tables IV, V, and VI, that $(NH_4)_2SO_4$ passes through the cellophane membrane in greater quantities than do NH_4Cl or NaCl. A correlation is evident when one compares the percent and moles of salt passing,

with the solubility of the salt. One thus concludes that the quantity of inorganic salt percrystallizing is somewhat dependant upon the solubility.

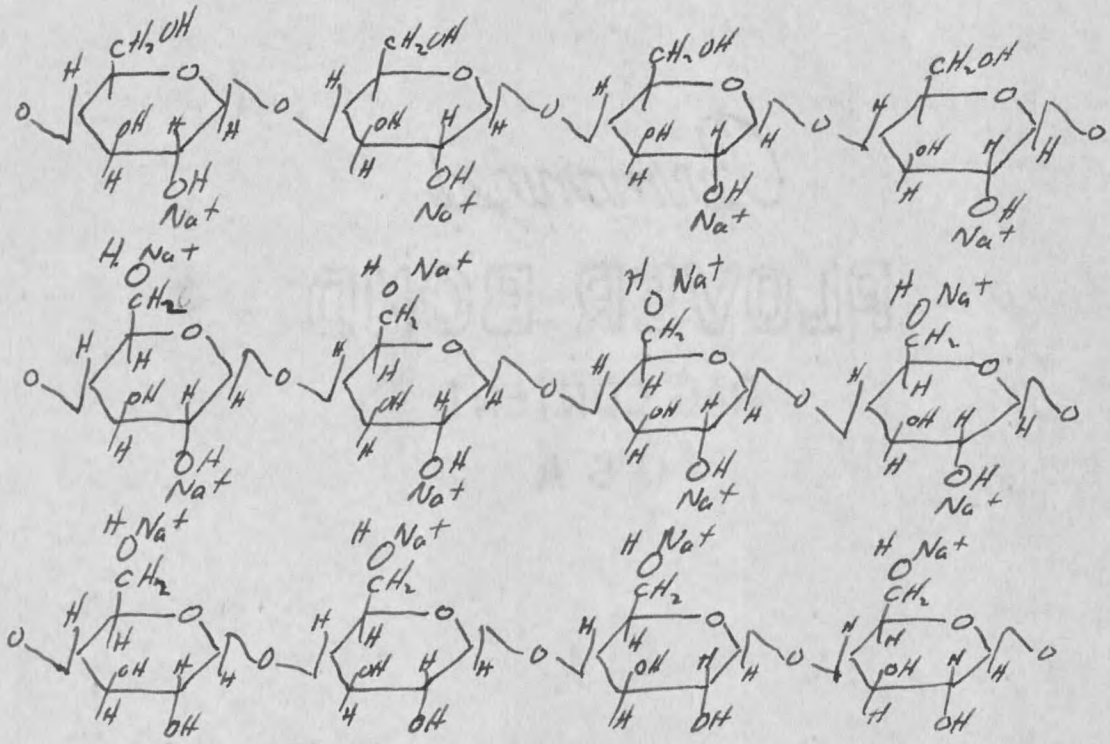
Several mechanisms can be offered as explanation for the percrystallization phenomenon. Inorganic salts ionize in solution and pass through a membrane in ionic form with the water as it evaporates through the lattice structure of the membrane. The anions and cations unite on the surface of the membrane and form crystals which build up to a considerable size. Some of the amino acid molecules form zwitterions, unite with anions or cations, and pass through the membrane with them. Zwitterions are large, and only a few get through the spaces in the lattice which is composed of cellulose units. The number of zwitterions passing through depends also upon the number of anions and cations passing through. As water evaporates through the membrane, the solution becomes more concentrated, reaching a point where the salt begins to crystallize on the inside. Little percrystallization will occur from this point on. Thus the quantity of percrystallization depends upon the solubility of the salt because percrystallization will practically cease when crystallization begins inside the membrane, and because percrystallization depends upon the number of ions formed and passed through the membrane.

Cellophane is produced from cellulose by the viscose process. The "viscose", cellulose xanthate, is forced through

fine slots into an acid bath producing thin sheets of regenerated cellulose. The structure remains basically the same, with B 1-4 glucoside linkage and the linear chains are probably linked together by hydrogen bonds.



Pigman (9) explains the effect of acids and salts on cellulose molecules as follows, "since the cellulose molecules probably are held in the micellar lattice by hydrogen bonds and Van der Waals forces, the solubilizing action of strong acids and salts probably is due to a replacement of bonds such as COHO by $\overset{H}{CONa}$."



"The action of acids and salts is accompanied by a degradation of chains due to hydrolysis of the glucosidic linkages. Mild hydrolytic conditioning leads to diminished chain length and loss of fiber strength."

Thus acids or salts rupture the lattice by replacing hydrogen bonds linking the chains, or by hydrolyzing glucosidic linkage. In the experimental work it was found that inorganic salts passed through the membrane from neutral solution, but amino acids and amino acid salts failed to percrystallize from acid solution, in the absence of inorganic salts. From this one must conclude that the inorganic salt or specifically the

cation has more effect on rupturing the lattice than the acid. It is more likely that the hydrogen bonds were disrupted by the cations than that the glucosidic linkages were hydrolysed. Evidence in support of this view is the fact that inorganic salts did not pass through collodion membranes. Collodion is pyroxylin, a nitrated cellulose, having NO_3 groups in place of some of the OH groups. Collodion membranes are more resistant to the effects of acidic or basic solutions than cellophane. If cations rupture hydrogen bonds in passing through a membrane, it is easy to explain why it would be difficult for salts to pass through collodion. However, if cations hydrolyse glucosidic linkages in passing through the membrane, it is difficult to explain why salts did not pass through a collodion membrane.

Conclusive evidence that the moleculer lattice of the cellophane membrane was affected to some degree is found in the fact that after an encrustation was removed from a membrane, tiny beads of liquid formed on the surface of the membrane. Evaporation soon took place and encrustation was noted. Percrystallization took place much more slowly on newly hung membranes and no noticable liquid could be observed on the surface. Thus the liquid droplets came through the enlarged spaces in the membrane caused by the rupture of linkages in the lattice by one of the mechanisms described above.

A greater percentage of tyrosine passed from each of the

three salt solutions than did cystine. This is consistant with what we know about the two acids. Tyrosine has a molecular weight of 181 as compared to cystine with a molecular weight of 240. The tyrosine molecule is more compact than the cystine molecule. Tyrosine is soluble in water to the extent of .041 grams per 100 ml. of water. Cystine has a water solubility of .006 grams per 100 ml. of water at 20° c. Tyrosine has a higher ionization constant whether it be from classical, zwitterion, or acidic formulation of ionization. Thus ionizing to a greater degree and being smaller in size, the tyrosine molecule passes through the membrane in greater quantity than cystine.

Kober (7) stated that colloidal material will not pass through a membrane. There is a possibility that the amino acids in the protein digest were in part in the colloidal state. However, the fact that some cystine and tyrosine passed from the salt containing solutions proves that it could not all be in the colloidal state. That no percrystallization took place from pure, completely soluble amino acid solutions, further proves that colloidality was not the source of difficulty.

Much work must be done before the mechanism of percrystallization can be fully understood. Work should be done on all of the amino acids and other organic solids. In the field of amino acids, the work should be directed toward the water

soluble ones to avoid the use of acid or base which weakens the membranes and prevents work with individual amino acids and inorganic salts without contaminants. More rapid and convenient quantitative methods should also be sought. Additional work should be done with collodion membranes, in an effort to duplicate Kober's work and perhaps ultimately arrive at a useful quantitative or qualitative separation method for amino acids.

SUMMARY

1. Percrystallization is the crystallization of a solute on the surface of a membrane, after having passing through the membrane, with the evaporated water, from the solution within.

2. Under the conditions described in this study, no percrystallization took place from any type of solution in a collodion membrane.

3. No percrystallization occurred on cellophane membranes containing inorganic salt-free protein digests.

4. No percrystallization occurred on cellophane membranes containing amino acid salts such as lysine-hydrochloride, cystine-hydrochloride, and the sodium salt of cystine.

5. Measurable quantities of cystine and tyrosine passed through membranes containing protein digests and inorganic salts such as $(NH_4)_2SO_4$, NH_4Cl, and NaCl.

6. The greater the quantity of inorganic salt passing through the membrane, the higher the percentage of amino acid passed through the membrane. The quantity of inorganic salt passing through the membrane depends primarily on the water solubility of the salt.

7. A slightly higher percentage of tyrosine passes through the membrane than cystine, as is expected because tyrosine has a lower molecular weight and a higher ionization constant.

8. Although the exact mechanism of percrystallization is unknown it is believed to envolve the rupture of hydrogen bonds in the lattice structure of the cellophane membrane by anions and cations of the inorganic salt. Zwitterions probably associate with the anions and cations, and are carried through the membrane with them. On the surface the anions and cations unite to form crystals.

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ACKNOWLEDGEMENT

The author takes this opportunity to express his deep appreciation to Dr. B. L. Johnson, under whose guidance this work was done, and to Dr. Lawrence O. Binder and Dr. Kenneth J. Goering for their help in the writing of the thesis. Appreciation is also expressed to Dr. Edward W. Anacker, Dr. Ray Woodriff, and Graeme Baker for their assistance.