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A CRYSTALLINE POLYPEPTIDE FROM THE SEED OF *CRAMBE ABYSSINICA*

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Abstract—A polypeptide with prolamine-like solubility was crystallized from an aqueous acetone extract from seed of *Crambe abyssinica* Hochst ex R. E. Fries. The name "crambin" is proposed for this polypeptide. Electrophoresis and sedimentation equilibrium show that it is homogeneous and has a mol. wt. of 5000, which agrees with the minimum mol. wt. calculated from amino acid composition. Amino acid residues per molecule were threonine, alanine, and aspartic acid, five each; glycine, isoleucine, and proline, four each; cystine, three; arginine, serine, tyrosine, and valine, two each; and glutamic acid, leucine, and phenylalanine, one each. Three amide nitrogen residues were found. Cysteine, histidine, lysine, methionine, and tryptophan were absent. After performic acid cleavage of the disulfide bonds, crambin remained electrophoretically homogeneous and its mol. wt. did not change significantly. These data indicate that crambin consists of a single peptide chain with intramolecular disulfide crosslinks.

INTRODUCTION

DURING a compositional study on the seed of *Crambe abyssinica* Hochst ex R. E. Fries a crude crystalline precipitate was obtained when acetone was evaporated from an 80% acetone extract of the defatted seed meal. Recrystallization, solvent extraction, and adsorption of accompanying pigments yielded a white crystalline material (Fig. 1), polypeptide in nature, for which the name "crambin" is proposed.

RESULTS

Isolation and Chemical Composition

Details of isolation and purification are described under Experimental. The elemental composition of crystalline crambin (dry basis) is N, 16·13 ± 0·04%; S, 4·01 ± 0·01%; P, 0·0%; and ash 0·0–0·2%. Paper chromatography of an acid hydrolysate gave a number of ninhydrin positive spots showing presence of a polypeptide or protein. Yield of crystalline crambin varied from 0·25 to 0·50 per cent by weight of the hexane-extracted seed meal. It was estimated that the hexane-extracted seed contained 0·5–0·9 per cent of the material. Extraction of 1 g samples of the seed coat, cotyledon, and hypocotyl parts of the seed indicated that the polypeptide was only present in the latter two tissues. The X-ray pattern of the material in contact with its mother liquor (Fig. 1) shows sharp bands indicative of a high degree of crystallinity. After air drying the absence of many of these bands shows loss of crystallinity with drying (Fig. 2).

Solubility

Crambin dissolved readily in absolute ethanol and in aqueous solutions of ethanol and of acetone which contained 50 per cent or more of the organic solvent. It dissolved slowly in

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0·1 N sodium hydroxide, but the odor of hydrogen sulfide on standing indicated decomposition of cystine. It showed some solubility in 8 M guanidine hydrochloride.

Based on qualitative tests in which 15 mg of sample was suspended in 10 ml of solvent, crambin showed no apparent solubility in cold or boiling water, dilute or 6 N hydrochloric acid, 3 M urea, 0·02 M aluminium lactate, 0·1 N acetic acid, 0·5 N sodium chloride, and 100% acetone. After crambin was treated with performic acid to cleave disulfide bonds[1] oxidatively it became soluble in water and dissolved more rapidly at neutral than acid pH.

Amino Acid Composition

The amount of each amino acid, expressed in different terms including residues per minimum mol. wt. of 4700 is given in Table 1. No lysine or histidine peaks were found.

TABLE 1. AMINO ACID COMPOSITION OF CRAMBIN

Amino acid	Grams amino acid from 100 g dry polypeptide*	Grams amino acid residues in 100 g dry polypeptide	Grams amino acid N per 100 g polypeptide N	Amino acid residues per 4700 g polypeptide
Aspartic acid	12·75 ± 0·70	11·04	8·31	4 6
Threonine	15·11	12 80	11·02	5·2
Serine	5 24	4·34	4 33	2·4
Proline	10·44 ± 0·20	8·78	7·88	4·3
Glutamic acid	2·95 ± 0·06	2·59	1·74	1·0
Glycine	6·17 ± 0·32	4 69	7·14	3·9
Alanine	8·94 ± 0·40	7·12	8·72	4 8
Valine	4·61	3·92	3·42	1 9
Cystine	14·15	12 05	10·22	2 8
Isoleucine	11·95	10·32	7·91	4·3
Leucine	3·86 ± 0·06	3·34	2·55	1·4
Tyrosine	7·21 ± 0·06	6·50	3·45	1·9
Phenylalanine	3·41 ± 0·12	3·04	1·80	1·0
Arginine	6·71 ± 0·14	6·02	13·37	1·9
Ammonia or amide nitrogen	--	--	6 70	3·0†
Total	113·4	96·6	98 6	—

* Average of at least four analyses except as follows: serine, threonine, and ammonia nitrogen extrapolated to hydrolysis time of zero from 18, 24, 72, and 120 hr acid hydrolysis; valine and isoleucine average of 72 and 120 hr hydrolyzates including the assigned *allo*-isoleucine as a part of the isoleucine; cystine average of two analyses.

† Amide nitrogen by method of Leach and Parkhill.[9]

Cysteine was absent, based on the nitroprusside test in the presence of guanidine hydrochloride as described by MacDonnell *et al.*[2], nor was there any tryptophan as determined by the method of Spies and Chambers.[3] No degradation products of tryptophan were observed from the ion-exchange analysis of the acid hydrolyzate. Ultraviolet absorption curves from a 60% ethanol solution of crambin showed a maximum at 277 mμ in Ca 0·1 N HCl and at 295 mμ in Ca 0·1 N NaOH (tyrosine) with no evidence of tryptophan absorption at 282 mμ.

The 18 hr acid hydrolyzate, when analyzed for amino acids using ion-exchange chromatography, gave a small peak in the position of methionine equal to 0·3 g methionine per 100 g

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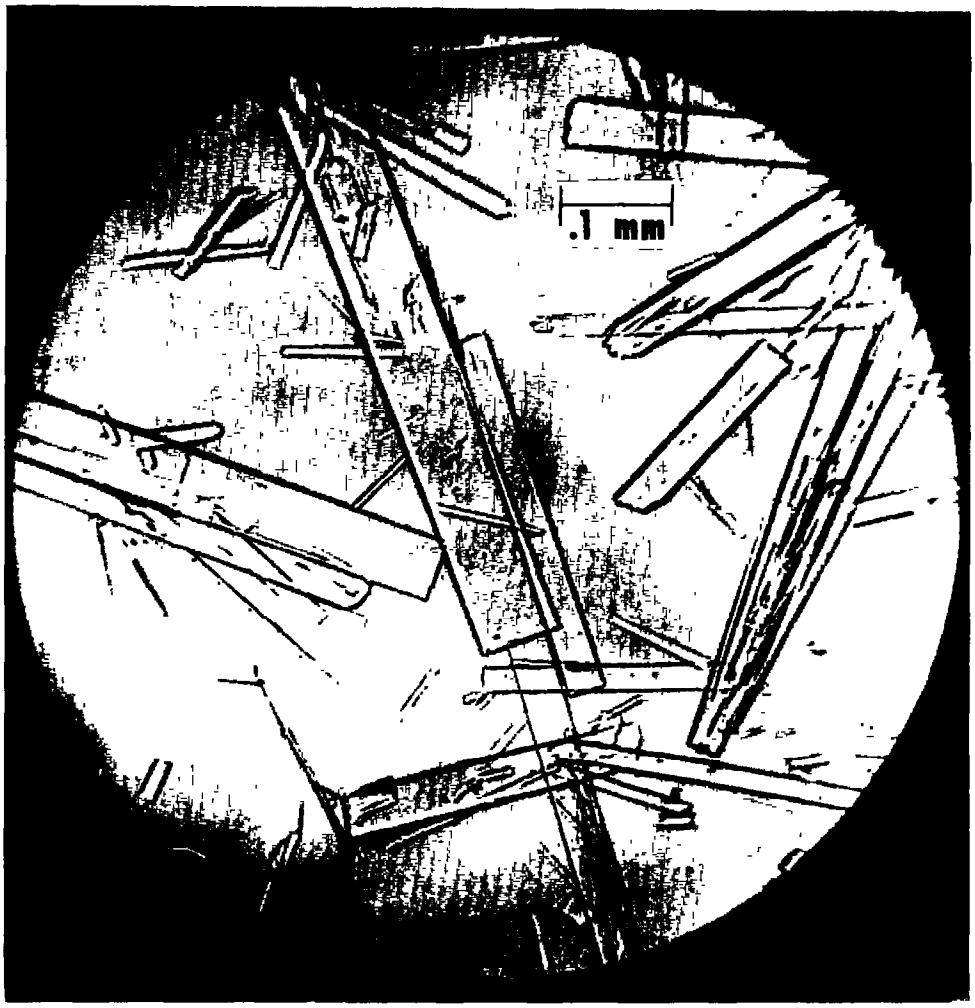

FIG. 1. CRYSTALLINE CRAMBIN FORMED BY EVAPORATION AT ROOM TEMPERATURE OF ALCOHOL FROM 50% AQUEOUS SOLUTION.

Magnification = ×150

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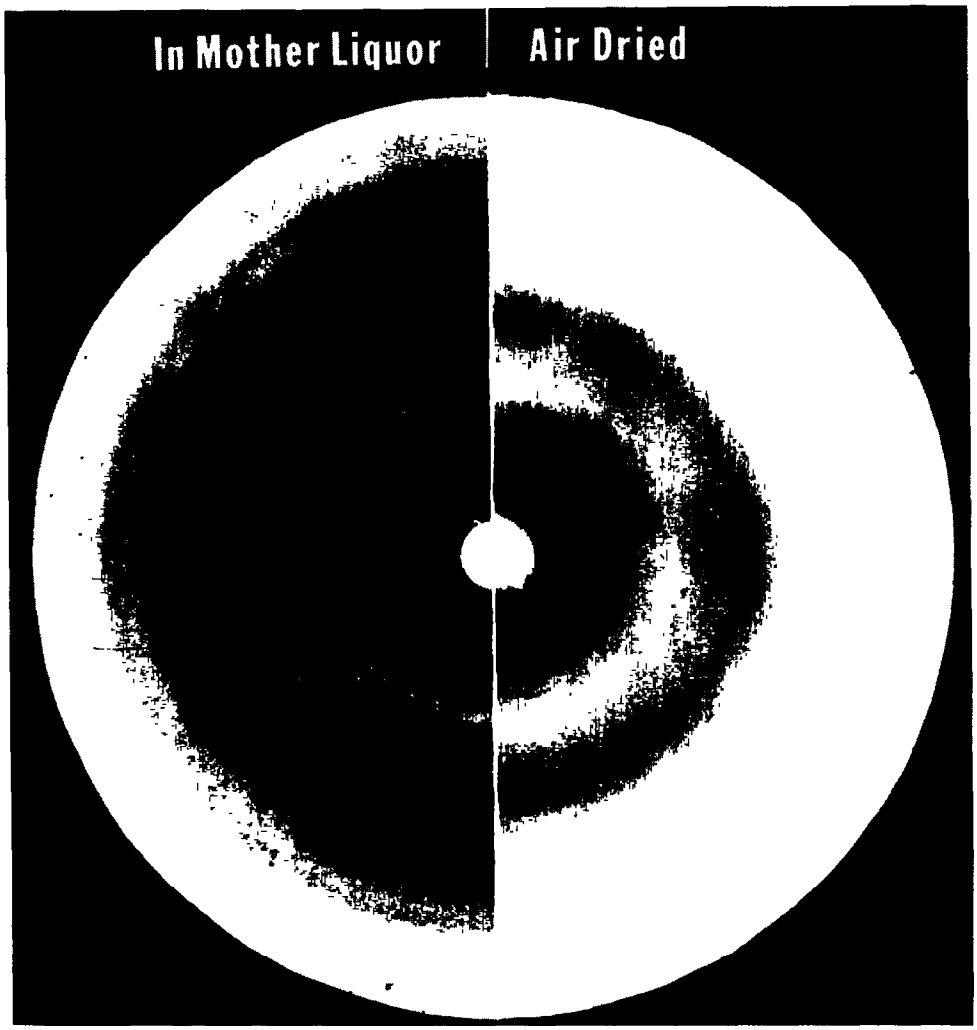

FIG. 2. X-RAY PATTERNS OF CRYSTALLINE CRAMBIN

protein. This peak increased in size with increased hydrolysis time to 0·8 g apparent methionine per 100 g of polypeptide in the sample when hydrolyzed 120 hr. However, paper chromatography of a hydrolyzate showed that crambin contained no methionine. The chromatogram was developed with *n*-butanol:ethanol:water (4:1:4), upper phase, and sulfur-containing amino acids were detected with platinum chloride, acid potassium iodide reagent.[4] Positive controls for this test were run on the hydrolyzate with methionine added and on hydrolyzates of protein known to contain methionine.

This apparent hydrolytic decomposition product eluting in the position of methionine is not any one of a large number of ninhydrin color-producing compounds, the elution positions of which have been reported by Zacharius and Talley.[5] Recently bis-(2-amino-2-carboxyethyl) trisulfide was identified as a decomposition product of cystine in acid hydrolyzates of wool.[6] It was thought that this compound might elute in the position of methionine. Ion-exchange chromatography of an authentic sample showed the compound eluted as a double peak under isoleucine. Pure cystine was hydrolyzed 18 hr and subjected to amino acid analysis to see if a decomposition product formed that eluted in the position of methionine. The only decomposition product found was bis(2-amino-2-carboxyethyl) trisulfide present as 2 per cent of the original cystine. Groves[7] suggested that *allo*-isoleucine on the 30–50° ion-exchange column should elute with methionine. On the 50° column *allo*-isoleucine elutes near methionine.[8] Ion-exchange chromatography at 30–50° of a mixture of authentic methionine, *allo*-isoleucine and isoleucine showed the *allo*-isoleucine eluted with methionine. Authentic isoleucine refluxed with 6 N hydrochloric acid under our conditions of acid hydrolysis of crambin gave a small peak in the position of methionine after 70 hr which became larger after 120 hr. On the basis of these results the peak eluting in the position of methionine was considered to be *allo*-isoleucine formed from isoleucine during the acid hydrolysis. However, no evidence was found in the literature that isoleucine forms *allo*-isoleucine in acid hydrolysis of a protein.

Because of likely error in the determination of amide residues from the ammonia formed during the acid hydrolysis of the peptide the amide residues were calculated from the ammonia formed by hydrolysis with 2 N hydrochloric acid at 100° as described by Leach and Parkhill.[9]

Ion-exchange chromatography of the acid hydrolyzates gave no indication of levulinic acid or related products formed from the acid hydrolysis of carbohydrates.[10] No positive test for carbohydrate was obtained on acid-hydrolyzed and unhydrolyzed crambin with alkaline silver reagent[11] after paper chromatography in *n*-butanol:ethanol:water (4:1:4) upper phase.

Molecular Weight and Electrophoretic Properties

The slow passage of crambin in 60% ethanol through cellulose dialysis tubing (about 90 per cent in 8 days) indicated a relatively small molecular size. The amino acid composition of the dialysate was the same as that of the residue which did not pass through the membrane.

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[9] S. L. Leach and E. M. J. Parkhill, *Proceedings of International Wool Textile Research Conference*, Australia, p. C-92 (1955).
[10] R. M. Zacharius and E. A. Talley, *J. Chromatog.* **7**, 51 (1962).
[11] I. Smith, *Chromatograhic Techniques*, p. 169, Interscience, New York (1958).

Sedimentation equilibrium analysis indicated a mol. wt. of 5000 and there was no evidence of polydispersity in a solvent system of 80% ethanol, 0·1 M potassium chloride, and 0·02 M hydrochloric acid. In the same solvent system at 25·3° a diffusion constant of $8{\cdot}20 \times 10^{-7}$ was obtained for crambin from the rate at which the system approaches equilibrium.

After cleaving of the disulfide bonds by performic acid, the mol. wt. was determined by sedimentation equilibrium in two aqueous systems. In 0·1 μ pH 8·0 tris buffer (0·2 M tris (hydroxymethyl) aminomethane plus 0·1 M hydrochloric acid) it exhibited a weight-average mol. wt. of 6400 and in 4 M urea plus 0·1 μ citrate (0·1 M monosodium citrate adjusted to pH 3·0 with citric acid) it exhibited a weight-average mol. wt. of 5700. (In the tris system the diffusion constant was $12{\cdot}82 \times 10^{-7}$ at 25°.) Some heterogeneity was observed in each system. In both the mol. wt. extrapolated to about 5000 at zero concentration. This behavior indicates some aggregation of a species of about 5000 mol. wt. rather than presence of larger covalently bound species in the systems.

Electrophoretically, the polypeptide showed only one band on cellulose acetate strips in a system of 60% ethanol plus 0·05 M citric acid plus 0·05 M sodium citrate (pH 3·9), and in a system of 60% ethanol plus 0·05 M HCl (pH 1·65). Moving boundary electrophoresis of performic acid-oxidized crambin at 1·0° in 0·05 μ tris pH 8·0 showed one peak with a mobility of 5·7 Tiselius units.

DISCUSSION

The best known prolamines are those of the cereal grains (Gramineae). Of these, wheat and corn prolamines have been most intensively studied.[12–14] Both gliadin and zein are mixtures of related components; contain large amounts of glutamine and proline; possess both inter- and intra-molecular disulfide bonds in fairly small amounts; comprise a major portion of the seed protein, and their mol. wt. are in the 20,000–50,000 range. In contrast, crambin is fairly easily isolated in pure crystalline form from an aqueous acetone extract of crambe seed; contains little glutamine or glutamic acid but large amounts of proline; contains a large amount of intramolecular disulfide bonds; and has a mol. wt. of 5000. The high cystine content of crambin is like that of some water-soluble proteins isolated from seeds. Proteins of high cystine content present, in most cases comprising a small percentage of the total seed protein, have been reported from barley,[15] cottonseed,[16] lima beans,[17] oats,[18] ground nut,[19] and wheat.[20]

Amino acid composition (Table 1) shows 96·6 g of amino acid residues per 100 g of dry polypeptide and 98·6 per cent of the nitrogen accounted for. Amino acid residues are close to whole integral numbers except for aspartic acid, serine, proline, isoleucine, and leucine. These five amino acids may not be as accurately measured by the method of analysis or some of them may be converted in part during the hydrolysis to other products. Of the 4·01 per cent sulfur, 94 per cent was found in the cystine.

[12] Y. V. WU and R. J. DIMLER, *Arch. Biochem. Biophys.* **102**, 230 (1963).
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[14] JAMES E. TURNER, JOYCE A. BOUNDY and R. J. DIMLER, Unpublished data.
[15] ROBERT JENSEN, *Acta Chem. Scand.* **6**, 771 (1952).
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Water-insolubility of crambin is probably due to its lack of charged groups (Table 1). Its solubility in 80% ethanol, we believe, is due to a combination of its low mol. wt. and its preponderance of hydrocarbon and hydroxyl-containing side chains. Performic acid cleavage of the disulfide bonds adds six charged sulfonic acid groups to the molecule, which probably account for solubility of the oxidized crambin in aqueous systems.

From diffusion constants obtained from the rate of approach-to-sedimention equilibrium, a frictional ratio of 1·27 was calculated for native crambin and about 1·46 after disulfide cleavage. The frictional ratios indicate a compact and symmetrical molecule[21] which unfolds somewhat after disulfide cleavage. This behavior is what one would expect for a crystalline polypeptide with a large number of intramolecular disulfide bonds.

It is unlikely that crambin was formed from cleavage of larger molecular units during isolation since mild conditions were used. On acid hydrolysis of the polypeptide no evidence was found of substances other than amino acids. Accordingly, it may be classified as a simple polypeptide of natural origin.

Since the polypeptide contains large amounts of sulfur as cystine, it may be in some way related to the large amount of thioglucosides in the seed of the plant.[22] Possible relationships of crambin to the enzyme system myrosinase (hydrolysis of thioglucosides) or to possible biological functions or effects have not been studied.

EXPERIMENTAL

Isolation and Purification

Mature seed (grown in Texas, 1961 crop) with the pericarp removed was flaked and cold hexane-extracted. The defatted seed ground to pass a 100-mesh screen was extracted with 80% v/v acetone water. In a typical preparation 165 g of the air-dry, defatted seed meal was extracted with 1600 ml of the aqueous acetone for 1 hr on a shaker followed by centrifugation and re-extraction twice more with 800-ml volumes of solvent. The combined supernatants were reduced in volume under vacuum at below 50° until the acetone was removed at which point crude crambin crystals precipitated. After standing overnight in the refrigerator, the crystals were separated by centrifugation. The centrifugate was dissolved in 200 ml of 67% v/v ethanol and filtered, if required, to remove insoluble gums. The yellow solution was extracted in a separatory funnel with an equal volume of petroleum ether. Five or six extractions were made which removed nearly all the color, ethanol being added to maintain the concentration in the polar phase. The polar phase was concentrated under vacuum to about 70 ml volume and 350 ml of absolute ethanol was added. The clear yellow solution was passed through an alumina column (adsorption alumina, Fisher Scientific Co., Cat. No. A-540)* 20 × 3 cm followed by 250 ml of 60% ethanol. The combined effluents were concentrated under vacuum below 50° until the crambin crystallized. After 16 hr at 0° the crystals were separated by centrifugation. Recrystallization in this manner was repeated (usually three or four times) until white crystals were obtained from a water-clear mother liquor. Yield: 0·43 g dry crambin. The petroleum ether extraction and passage through the alumina separated pigments which could also be removed by extensive recrystallization.

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* The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Sedimentation and Electrophoretic Studies

Sedimentation equilibrium analysis was done in a Spinco Model E ultracentrifuge (equipped with a rotor temperature indicator and control system): with 30 mm double sector cells for equilibrium runs and capillary synthetic boundary cells for initial concentration determinations. For concentration determinations dn/dc values were calculated from the equation:[23]

$$\frac{dn}{dc} = 0{\cdot}01\bar{v}_p(n_p - n_o)$$

in which $\bar{v}_p$ is the partial specific volume of the peptide, n_p is the refractive index of the peptide, and n_o the refractive index of the solvent. A partial specific volume value of 0·719 was calculated from amino acid analysis by a procedure given by McMeekin *et al.*[24] and a refractive index of 1·6064 was calculated from amino acid analysis by another procedure of McMeekin *et al.*[25] Diffusion constants were obtained from the rate at which the system approached equilibrium by the procedure of Van Holde.[26,27] Other calculations were done by standard procedures.[28]

Electrophoresis on cellulose acetate was done on Sepraphore III strips according to procedures supplied by the Gelman Instrument Co., the manufacturer of the apparatus. Moving boundary electrophoretic analyses were made with a Spinco Model H instrument.

Ion-Exchange Chromatographic Amino Acid Analysis

Acid hydrolysis was carried out under reflux on 25-mg samples in 50 ml of twice distilled 6 N hydrochloric acid. The hydrochloric acid from the water-clear hydrolyzate was removed under vacuum at less than 50° and the residue made to a convenient volume with pH 2·2 buffer for analysis by the ion-exchange chromatographic method of Spackman *et al.*[29] with a Beckman Spinco automatic instrument. Cystine was oxidized to cysteic acid by the method of Schram *et al.*[30] and determined on the 150-cm column of the analyzer. Analytical values by this method were essentially the same as from the cystine plus meso-cystine peaks from acid hydrolysis of unoxidized crambin.

Dialysis Through a Cellulose Membrane

Standard cellulose dialysis tubing prepared by the viscose process, 1⅝ in. flat width (A. H. Thomas and Co., No. 4465-A2), was used for the membrane in 60% ethanol as solvent. In a typical experiment 150 mg of crambin in tubing having a 50-ml volume was dialyzed for 206 hr against 500 ml of solvent changed twice daily. Estimation of the weight of crambin that had passed through the membrane was: 35, 58, 73, 89, 102, and 108 mg at 24, 48, 72, 96, 139, and 182 hr, respectively. After 206 hr, 19 mg of solids remained inside the membrane.

Ultraviolet absorption curves were obtained from a Cary recording spectrophotometer.

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Micro-Kjeldahl nitrogens, sulfur, phosphorus, and ash determinations were run essentially as described by Steyermark.[31]

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