corresponding alcohol, tryptophol (IV), by the excellent method of Nystrom and Brown.[9] An ether solution of III was treated with lithium aluminum hydride, suspended in ether, to give IV in good yield (65%).

## Experimental

**3-Indoleacetic Acid (III).**—Gramine (II) was prepared by the Mannich reaction on indole (I) according to the method of Külin and Stein.[7] A mixture of 25.0 g. of gramine (II), 35.2 g. of sodium cyanide, 280 ml. of 95% ethyl alcohol and 70 ml. of water was boiled for eighty hours. To the cooled reaction mixture was added 350 ml. of water. The solution was treated with Norite, filtered, concentrated under reduced pressure until all the alcohol had been removed, cooled to 5° and filtered. The solid on the funnel (7.0 g., m. p. 145–150°) was recrystallized from alcohol and ether to give 5.0 g. of 3-indoleacetamide, m. p. 149–151°. A sample of the amide, prepared for analysis by recrystallization from absolute alcohol and petroleum ether, was found to melt at 153°. The amide has been previously reported to melt at 150–151°.[10,5]

*Anal.* Calcd. for $C_{10}H_{10}N_2O$: N, 16.09: Found: N, 15.94.

The reaction mixture, after removal of amide by filtration, was concentrated under reduced pressure to a volume of approximately 300 ml. and cooled to 10°. Dropwise addition of cold, concentrated hydrochloric acid (hood!) to the vigorously stirred solution caused precipitation of crude, slightly pink 3-indoleacetic acid. The crude material was filtered and dried at 70°; yield 20.0 g. (79.5%) of product melting at 158–161°. This material was recrystallized from ethylene dichloride containing a small amount of alcohol to give pure 3-indoleacetic acid (III), m. p. 167–168°; yield 17.4 g. (69.2%). A sample of III, recrystallized from ethylene dichloride was found to melt at 168–169° (lit. m. p. 164.5–165°).[5]

A solution consisting of 1.0 g. of 3-indoleacetamide (m. p. 149–151°), 1.2 g. of sodium hydroxide and 8.0 ml. of water was boiled for four hours. The cooled (5°) solution was treated with Norite, filtered and made strongly acidic (*p*H about 1.5) with concentrated hydrochloric acid. The 3-indoleacetic acid which precipitated was collected on a Büchner funnel and dried at 70°. The yield of product, melting at 167–168°, was 0.95 g. (95%). On the basis of this experiment the over-all yield of pure III from gramine (II) was 88%.

In one experiment the alkaline solution of 3-indoleacetic acid was treated with acid to adjust the *p*H to about 4.5 (instead of 1.5). The product which precipitated proved to be a mixture of III and the sodium salt of III. In those cases where the *p*H was adjusted to about 1.5 the product was found to be only III.

**β-(3-Indole)-ethanol (Tryptophol) (IV).**—To a rapidly stirred suspension of 1.4 g. of lithium aluminum hydride in 100 ml. of anhydrous ether was added a solution of 5.0 g. of III in 300 ml. of anhydrous ether at such a rate that gentle reflux was maintained. The reaction mixture was stirred for fifteen minutes after the addition was completed. The excess lithium aluminum hydride was decomposed by the dropwise addition of a few milliliters of water. The reaction mixture was treated with 60 ml. of 10% sodium hydroxide solution (added dropwise), and the ether layer was separated; the aqueous layer was filtered, and the filtrate was extracted with 100 ml. of ether. The combined ether solutions were evaporated under reduced pressure. The residual oil did not readily crystallize and was distilled to give 3.0 g. of tryptophol (IV), b. p. 174° (2 mm.); yield 65.2%. Some decomposition of the product occurred during the distillation. On standing the oily product crystallized to give solid material melting at 57–58°; the mixed m. p. with an authentic sample of IV, prepared by another method,[3] was 57–58°.

## Summary

Tryptophol [β-(3-indole)-ethanol] has been prepared *via* the sequence: indole, gramine, 3-indoleacetic acid, tryptophol. Detailed experimental conditions are given for the preparation of 3-indoleacetic acid from gramine.

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(9) Nystrom and Brown, THIS JOURNAL, **69**, 1197 (1947); **69**, 2548 (1947).

(10) Baker and Happold, *Biochem. J.*, **34**, 657 (1940).

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[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEARCH LABORATORIES, AMERICAN CYANAMID COMPANY]

# Chemical Studies on Polymyxin.[1] I. Isolation and Preliminary Purification

BY R. G. SHEPHERD, P. G. STANSLY, R. WINTERBOTTOM, J. P. ENGLISH, C. E. FELLOWS, N. H. ANANENKO AND G. L. GUILLET

In a previous report[2] describing the chemotherapeutic properties of polymyxin, the isolation of the antibiotic mixture from the fermentation liquor of *Bacillus polymyxa* was briefly described. The procedure employed was based on charcoal adsorption, elution with acid methanol, and precipitation of the hydrochloride salt with acetone. To facilitate investigation of this antibiotic, the present communication describes its isolation and purification in detail. Some of the properties of polymyxin are also summarized. The preparation of polymyxins which are homogeneous with respect to biological activity and the identity of the constituents are reported in a separate paper.[3]

## Experimental

The method of assay used in these isolation studies and the definition of the "unit" of activity have been reported earlier.[4] The assay error of any single determination has been estimated to be ±15%. Except as otherwise stated, the various comparative experiments were carried out on 100–500 cc. quantities of fermentation liquor prepared

(1) Polymyxin is a generic term for the antibiotics (first isolated from *B. polymyxa* filtrates) which have the closely related biological and chemical characteristics described elsewhere (refs. 2 and 3). This paper was presented before the Division of Biological Chemistry at the American Chemical Society meeting in Chicago on April 20, 1948.

(2) Stansly, Shepherd and White, *Bull. Johns Hopkins Hosp.*, **81**, 43 (1947).

(3) Bell, *et al.*, *Ann. N. Y. Acad. Sci.*, **51**, in press (1948).

(4) Stansly and Schlosser, *J. Bact.*, **54**, 585 (1947).

from the "standard" medium[5] or, in the subsequent steps, on an equivalent amount of activity.

**Clarification of Fermentation Liquor.**—Clarification was carried out prior to adsorption in order to remove insoluble matter and to prevent contamination with another antibiotic retained by the cells of *B. polymyxa*.[6] The use of Hyflo Supercel was superior to centrifugation or treatment with flocculating agents on the basis either of the resulting clarity or loss of activity. By stirring 2 g. of Hyflo Supercel per 100 cc. into the fermentation liquor and using an equal amount as a filter pre-coat, a satisfactory rate of filtration and complete clarification were obtained without appreciable loss of activity. The clarificate was a clear yellow-brown liquid having a *p*H of 6.5.

**Adsorption of Activity.**—At a level of 10 g. per 100 cc. of clarificate, a series of siliceous earths consisting of Celite 545, Hyflo Supercel, Standard Supercel, Filtercel and Superfiltrol showed increasing adsorption with decreasing particle size. Since the pyridine–acid mixtures required for elution did not yield satisfactory concentrates, attention was directed to the charcoals. Darco G-60, Darco S-51 and Norit A at 0.5 g. per 100 cc. gave complete adsorption of polymyxin from fermentation liquor containing 250 units per cc., while Nuchar required 2 g. per 100 cc. and filtered very slowly. The per cent. elution from all four was approximately the same and Darco G-60 was arbitrarily chosen for use.

To determine the optimal conditions for adsorption and elution using Darco G-60, replicate experiments were carried out varying the amount of adsorbent. Fermentation liquors assaying about 250 units per cc. were used for this purpose. The quantity of charcoal in g. per 100 cc. (first figure) and the average per cent. adsorption (second figure) were 1, 100; 0.8, 100; 0.75, 100; 0.6, 100; 0.5, 100; 0.4, 100; 0.3, 94; 0.25, 80; 0.2, 72; 0.1, 48; 0.06, 45; 0.03, 30; and 0.016, 30. A downward trend of the average per cent. elution with acidified alcohols was observed when excess amounts of Darco G-60 were used. The average recovery of activity was highest at 0.2–0.4 g. of charcoal per 100 cc. and the latter quantity was selected for routine use.

**Treatment of Charcoal after Adsorption.**—The charcoal was washed successively with water, 50% ethanol and absolute methanol with a very small loss of activity (< 1%). The 50% ethanol was found to be a very selective wash, use of which raised the potency of the concentrate from 200 to 1400 units per mg. When the ethanol concentration was varied, 50% ethanol gave a definite maximal removal of color and inert solids from Darco G-60. Ethanol was found to be more effective than methanol or acetone for this purpose.

**Elution from Charcoal.**—Elution of polymyxin from Darco G-60 was negligible or very poor (< 3%) with the following solvents: water; 0.05 *N* hydrochloric acid; aqueous solutions of acetic acid, ammonia, pyridine or diethylamine; aqueous or absolute methanol or ethanol; and absolute pyridine or diethylamine. Solvents giving about 10–20% elution were: 80% ethanol containing sulfuric, phosphoric or acetic acids. The following gave 30–60% elution: 80–100% methanolic or ethanolic 0.05 *N* hydrochloric acid; glacial acetic acid; and 20% pyridine in 30% aqueous acetic acid. The acidified alcohols were chosen for use as the simplest eluants permitting direct precipitation of a polymyxin salt.

Of the acid alcoholic solvents (0.05 *N* hydrochloric acid), the greatest elution was obtained with 80% ethanol and 80% or absolute methanol. Absolute methanolic hydrochloric acid (0.05 *N*) was chosen for further work in order to minimize the concentration of water at the precipitation step. This gave 35–50% elution upon stirring for one-half hour at 25° with just sufficient eluant (one-thirtieth of the volume of the fermentation liquor) to form a slurry with the charcoal. This volume was used in order to minimize the subsequent precipitating volume. A twenty-fold increase in the volume of eluant produced a corresponding decrease in the concentration of activity (1.5% to 0.08%) without altering the per cent. elution.

In order to increase the amount eluted, various modifications of these conditions were investigated. Increasing the contact time from one-half to four hours or raising the temperature to the boiling point did not change the amount of activity eluted. Repetition of the elution after filtration and rinsing gave rapidly declining amounts of activity in a series of five successive eluates as follows: 40, 7, 4, 2 and 1%. Since each of these eluates gave solid concentrates of the same potency, they were subsequently pooled before precipitation of polymyxin hydrochloride.

**Precipitation of Polymyxin Hydrochloride.**—Addition of the acid methanol eluates to nine volumes of acetone was found to be a convenient means of quantitatively isolating the hydrochloride. This procedure had additional advantages over other precipitating solvents, such as ether and petroleum ether, in retaining large amounts of inert solids in solution and in being unaffected by 3–15% water in the eluate. Although fractional precipitation with acetone partially eliminated a Sakaguchi-positive impurity, the potency of the fractions were almost the same. Since this impurity was completely removed by the purification described below, the eluates were quantitatively precipitated in one step.

The polymyxin hydrochloride was generally recovered by centrifugation and washed successively with acetone and ether. After aspiration of the final ether wash liquid, the solid must be placed at once in a continuously evacuated desiccator over calcium chloride. If the moist solid comes in contact with air, it rapidly turns brown and liquefies. After drying, the powder is stable in air and is only slightly hygroscopic. Filtration of the precipitated solid can be carried out successfully only if the filter cake is *always* kept covered with mother liquor or wash liquid. The final ether wash is allowed to just reach the surface of the filter cake and the funnel is immediately placed in a continuously evacuated desiccator over calcium chloride.

**Procedure for Isolation on 10-Liter Scale.**—Ten liters of fermentation liquor having an activity of about 250 units per cc. and a *p*H of 6.6 was stirred with 200 g. of Hyflo Supercel and filtered on a large Buchner funnel coated with an equal amount of Hyflo. This filtration and the subsequent charcoal filtration are much more rapidly carried out by using a small (12 inch) Sparkler horizontal pressure filter. The activity (85–111% of orig.) and *p*H of the solution were unchanged by clarification.

The clear filtrate was then stirred for one-half hour with 40 g. of Darco G-60. The charcoal was recovered by filtration without the use of a filter-aid and washed on the filter with 5 liters of water. The filter cake was then stirred for a short time with 1 liter of 50% ethanol and refiltered. Washing was completed by two more treatments with the same volume of 50% ethanol, recycling each portion until the color no longer increased. A final rinse with absolute methanol was carried out to reduce the water content of the filter cake. The activity is stable to storage at room temperature in the adsorbed form.

The charcoal was eluted by stirring for one-half hour at room temperature with 350 cc. of absolute methanol containing 2.7 cc. of concentrated hydrochloric acid. The elution was repeated four times with the same volume of methanol containing 1.4 cc. of acid.

The pooled eluates (about 1.6 liters) were refiltered to remove a trace of charcoal and precipitated by addition to 14.5 liters of acetone with stirring. Polymyxin hydrochloride separated rapidly from the clear supernatant as a white flocculent precipitate. Occasionally, the supernatant solvent was turbid, in which case, precipitation was completed by addition of more hydrochloric acid (4 *N* solution in absolute ethanol).

The hydrochloride was recovered by aspiration of a large portion of the clear supernatant solvent and centrifugation of the residual slurry in 200-cc. bottles. The solvent was removed by aspiration and the solid washed twice with acetone and once with ether by suspending in the wash liquid. Immediately after removal of the ether wash, the

(5) Stansly, *et al.*, *J. Bact.*, **55**, 573 (1948).

(6) Stansly and Schlosser, *ibid.*, **54**, 549 (1947).

bottles were placed *in vacuo* over calcium chloride and evacuation continued until the solid was no longer visibly wet. The over-all recovery of activity on this scale of isolation was 30–60% and the product assayed 1100–1400 units per mg.

The results in Table I are typical of those obtained in the laboratory with 6–35 liter quantities of fermentation liquor.

TABLE I

POLYMYXIN ISOLATION

| Material | Amount | Units/cc. | Result |
|---|---|---|---|
| Ferm. liquor | 10 l. | 250 | *p*H 6.5–7.0 |
| Hyflo filtrate | 20 g./l. | | 100% orig. act'y |
| Darco filtrate | 4 g./l. | <2 | 98% adsorbed |
| Water wash | 1/2 vol. | <2 | 0.1% loss |
| 50% Alc. wash | 1/3 vol. | 2 | <1% loss |
| Eluate I | 1/30 vol. | 1900 | 45% elution |
| II | 1/30 vol. | 248 | 6% elution |
| III | 1/30 vol. | 93 | 3% elution |
| IV | 1/30 vol. | 40 | 2% elution |
| V | 1/30 vol. | 23 | 1% elution |
| Solid | | 1430 U/mg. | 98% recov. |
| Acetone filtr. | 1.5 vols. | *ca.* 2 | <2% loss |

**Other Isolation Methods.**—Although the charcoal adsorption–elution procedure was developed first and used most extensively, there are other satisfactory methods based on extraction or direct precipitation. For example, the activity was extracted with butanol after making the clarificate alkaline or after adding sodium chloride (20 g./100 cc.). The hydrochloride was then obtained by acid extraction of the solvent and vacuum freeze-drying.

Precipitation from neutral fermentation liquor with tannic acid (0.01 g./100 cc.) or from alkaline solution with benzaldehyde yielded solids from which the hydrochloride was obtained by solution in acid methanol and precipitation with acetone.

**Purification of Crude Polymyxin Hydrochloride. I. By Butanol Extraction from Alkaline Solution.**—One and a half grams of crude concentrate (assaying 1400 units per mg.) was dissolved in 100 cc. of distilled water. The solution was brought to *p*H 9.0 with 30 cc. of 0.1 *N* sodium hydroxide and extracted with 130 cc. of C.P. *n*-butanol. The phases were separated as rapidly as possible to minimize decomposition. The *p*H of the aqueous phase, containing most of the original color, had fallen to 8.5 and was readjusted to 9.0 with 3.8 cc. of 0.1 *N* alkali. This solution was immediately extracted again with 130 cc. of butanol and, after separation of the phases, the aqueous layer contained about 10% of the original activity. The *p*H (now 8.6) was readjusted with 1.8 cc. of 0.1 *N* alkali and a third extraction (130 cc.) carried out. The aqueous phase (now at *p*H 8.8) contained about 2% of the original activity.

The butanol extracts were combined and washed twice with 10 cc. of distilled water. The activity was quantitatively extracted from the butanol with three 80-cc. portions of 0.05 *N* hydrochloric acid. The aqueous phase was then extracted three times with 200-cc. portions of peroxide-free ether or petroleum ether and aerated with nitrogen to remove the organic solvent. The aqueous solution was then vacuum freeze-dried and subsequently redissolved and redried in order to obtain a fine powder, free from excess hydrochloric acid. The residue was a white powder assaying 1700–1800 units per mg. Polymyxin hydrochloride prepared in this way no longer gave the Sakaguchi reaction but was not ash-free.

**II. By Picrate Formation from Acid Solution.**—One gram of crude polymyxin hydrochloride (assaying 1400 units per mg.) was dissolved in 130 cc. of distilled water and treated with 0.18 g. of picric acid dissolved in 20 cc. of hot water. A small amount of crude oily picrate was separated by cooling and centrifugation. The main portion of picrate was prepared by addition of 0.895 g. of picric acid in 10 cc. of boiling absolute methanol. The chilled mixture was centrifuged and the picrate washed twice with saturated aqueous picric acid. After vacuum drying, the picrate was dissolved in 20 cc. of absolute methanol and precipitated with 200 cc. of absolute ether to remove excess picric acid. The solid was washed twice with ether and dried *in vacuo* to an orange-yellow powder. This material weighed 900 mg. and contained 94% of the original activity. The remaining activity (about 4%) in the mother liquor was recovered by saturation with picric acid (1.8 g. added as a slurry in 20 cc. of boiling methanol).

Polymyxin picrate prepared in this way has an activity of about 1100 units per mg. and is free from ash and chlorine. The salt melts with decomposition at about 200° and is levorotatory. The picrate is quite insoluble (< 0.001 %) in saturated aqueous picric acid but is considerably more soluble in distilled water. This salt is very soluble in acetone, methyl ethyl ketone, methanol and ethanol, and insoluble in chloroform, petroleum ether and ethyl ether.

Polymyxin picrate, 900 mg. dissolved in 9 cc. of methanol, was converted to the hydrochloride by acidification with 2 cc. of 4 *N* hydrochloric acid in absolute ethanol and addition of this mixture to 110 cc. of acetone with stirring. The precipitate was separated by centrifugation and washed twice with acetone and once with ether. After vacuum drying, the solid was reprecipitated twice in the same way using in the final precipitation only 0.2 cc. of the acid ethanol. The product was a white powder assaying 1700–1800 units per mg. and containing less than one part per million of picric acid. Both the Sakaguchi-positive and ash impurities are removed by this purification.

Separation into smaller fractions than described in the procedures above did not produce material of substantially higher potency. A superior product assaying 1800–1900 units per mg. resulted from the successive use of these two methods. Obtaining a further increase in potency and separation of the biologically active components of this product necessitated the use of partition chromatography.[3]

**Effect of Extraneous Substances on Assay.**—The assay of solutions involved in the chemical work on polymyxin brought to light two effects of extraneous substances on the size of the zone of inhibition on agar plates. One effect was the stimulation of growth of the assay organism, *E. coli*, by certain "toxic" substances, so that a smaller zone of inhibition resulted. Picric acid, butanol and other alcohols gave this effect but alone produced no zone of inhibition. A 0.01% polymyxin solution saturated with *n*-butanol had an apparent activity of 125 units per cc. when assayed directly but 200 units per cc. when assayed after vacuum evaporation.

The opposite effect was caused by the presence of various salts such as sodium chloride, calcium chloride, ammonium sulfate and potassium phosphate. Concentrated solutions of these salts produced no zone of inhibition of *E. coli* but caused considerable enlargement of the zone produced by polymyxin. A 0.013% polymyxin solution exhibited an apparent increase of activity from 260 units per cc. to 640 units per cc. in the presence of 20% sodium chloride.

**Properties of Polymyxin Hydrochloride.**—The purified salt prepared by the methods described above is an amorphous white powder whose decomposition point at 240° varies with the rate of heating. It is levo-rotatory in aqueous solution and gives the biuret and ninhydrin tests but not the Sakaguchi reaction. The hydrochloride is very soluble in water (> 40%) and methanol but shows a rapidly decreasing solubility in the higher alcohols. It is insoluble in ether, acetone, chlorinated solvents and the hydrocarbons.

This salt contains C, H, O, N, Cl (ionic) and is free from sulfur, alkoxy and ash. Its polypeptide nature is demonstrated by several observations. The total nitrogen (*ca.* 15%) is amino nitrogen after acid hydrolysis. The ninhydrin $\alpha$-amino carboxyl analysis shows a large amount of amino acid after hydrolysis. The unhydrolyzed material is a primary amine and contains no acidic groups demonstrable by the ninhydrin carboxyl method or by aqueous and formol titrations (acidity is equivalent to chloride content).

The stability of polymyxin to heat, acid, alkali and proteolytic enzymes has been discussed in earlier reports.[2,7]


## Summary

The preparation of polymyxin hydrochloride assaying 1000–1400 units per mg. is described in detail. The preferred method is by adsorption on Darco G-60, elution with acid methanol and precipitation with acetone. Purification of the crude hydrochloride and certain properties of polymyxin hydrochloride and picrate are described.

Related isolation studies and alternative methods of isolation are briefly discussed.


(7) Stansly and Ananenko, *Arch. Biochem.*, **15**, 473 (1947).

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[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

# Starch. II. Molecular Weights of Amyloses and Amylopectins from Starches of Various Plant Origins

BY A. L. POTTER AND W. Z. HASSID

It is now recognized that starch is not homogeneous but can be separated into two fractions, amylose and amylopectin, each having a different chemical constitution. The amylose consists of long unbranched chains, the amylopectin of chains with branches that are relatively short. There is no adequate information at present regarding the molecular weights of the two starch components. Much of the available information concerning molecular weights was derived from work on partly degraded acetylated or methylated starch, employing viscosity measurements.[1,2,3] The molecular weights inferred from such viscosity determinations can at best be considered only as rough approximations. Carter and Record,[4] using osmotic pressure measurements on whole starches, which were partly degraded with hot alcoholic hydrochloric acid and then methylated, obtained molecular weights ranging from 40,000 to 124,000. Osmotic pressure measurements of acetylated corn amylose and corn amylopectin in tetrachloroethane by Meyer, *et al.*,[5] gave molecular weights of about 78,000 and 300,000, respectively. Caesar, *et al.*,[6] applying Barger's method for the determination of the molecular weights of the nitric triester derivatives of starches in ethyl acetate, obtained a molecular weight of 360,000 for the potato amylose derivative and of 64,000 for the corn amylose derivative.

Inasmuch as amylose readily retrogrades from water solution and amylopectin forms a highly colloidal solution, molecular weight determination of these substances cannot be made in this solvent. It is therefore necessary to use the acetylated or methylated derivatives which are soluble in organic solvents. However, it is recognized that during the process of acetylation of starch with acetic anhydride at 60° in the presence of pyridine some degradation of the molecule occurs due to the elevated temperature. Similarly, partial degradation of starch occurs during methylation when this process is carried out in an alkaline medium, especially since it is necessary to repeat the process about eight times in order to completely methylate the product.

The purpose of this investigation was to determine the molecular size of the amylose and amylopectin fractions of starch under conditions of minimum degradation. The mildest possible treatment was therefore employed in the isolation of starch and the separation into its components. Intrinsic viscosity in 1 *N* potassium hydroxide was used as a criterion of degradation. Only starches with the highest viscosities were used.

In the preparation of the acetylated derivatives elevated temperatures were avoided. Using formamide as a dispersion medium[7] for the starch fractions, acetylation with a mixture of acetic anhydride and pyridine could be carried out at room temperature.

Periodate oxidation data[8] showed that the amylopectins from a number of starches of different plant sources ranged from 22 to 27 glucose units per end-group, while the chain lengths of the corresponding amyloses ranged from 420 to 980 glucose residues. In this connection it was of interest to find out whether these amyloses and amylopectins differed in their total molecular size. The determination of the molecular weight of amylose in conjunction with an end-group determination should also answer the question as to whether or not a single amylose molecule constitutes one chain, or whether several chains are combined to form the molecule.

The molecular weights of the starch fractions were determined by osmotic pressure measurements of the acetylated products in chloroform. Within the range of concentration used, the relationship of the osmotic pressure and concentration[9] could be expressed by $\pi/C = aC^n + b$, where

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(3) H. Staudinger and E. Husemann, *Ber.*, **71**, 1057 (1938).
(4) S. R. Carter and B. R. Record, *J. Chem. Soc.*, 664 (1939).
(5) K. H. Meyer, P. Bernfeld and W. Hohenemser, *Helv. Chim. Acta*, **23**, 885 (1940).
(6) G. V. Caesar, N. S. Gruenhut and M. L. Cushing, THIS JOURNAL, **69**, 617 (1947).
(7) J. F. Carson and W. D. Maclay, *ibid.*, **68**, 1015 (1946).
(8) A. L. Potter and W. Z. Hassid, *ibid.*, **70**, 3488 (1948).
(9) I. S. and E. S. Sockolnikoff, "Higher Mathematics for Engineers and Physicists," McGraw-Hill Book Co., New York, N. Y., 1941, p. 533.