The Synthesis of Some Aromatic Sulfonic Acids

and

A Procedure for the Purification of L(-) Leucine

Thesis by

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In Partial Fulfillment of the Requirements for the Degree of

Master Of Science

California Institute of Technology

Pasadena, California

1 9 4 7

Acknowledgement

The author wishes to take advantage of this opportunity to thank Professor Carl Niemann for his helpful assistance and advice during the progress of this research.

Introduction

Of fundamental interest to the protein chemist is the establishment of the composition of the various proteins in terms of the constituent amino acids, and also the isolation of these amino acids in a highly purified form. Many techniques have been proposed for this purpose, and among the more recent is the use of the aromatic sulfonic acids as specific precipitants.

Precipitation of the basic amino acids by aromatic sulfonic acids has repeatedly been reported in the literature. About forty years ago, Suida (1) described the salts of basic amino acids with two sulfonated dyes, orange II and crystal Ponceau. More recently, Kossel and Gross (2) suggested the use of flavianic acid for the isolation and determination or arginine and other bases. Zimmerman (3) obtained salts of quinizarinsulfonic acid with basic substances and with tyrosine and phenyl alanine, and Ackermann (4) noted that the basic amino acids were precipitated by 2, 6-diiodophenol-4-sulfonic acid.

The aromatic sulfonic acids are substances so strongly acidic that they may be expected to form salts with all types of amino acids, and this expectation was well verified in the case of flavianic acids by Crosby and Kirk (5), who prepared and described the microscopic characteristics of fourteen crystalline amino acid flavianates. However, it was not generally recognized that many of the sulfonic acid salts of the neutral monoamino acids were sparingly soluble, and until the recent work of Bergmann and coworkers (6,7,8,9), the mono- and diflavianates of arginine, which at 0° C have solubility products of $1{\cdot}3 \times 10^{-7}$ and $2{\cdot}4 \times 10^{-8}$ respectively, were the only salts that had come into general use.

Bergmann *et al.* made an extensive investigation of the amino acid salts of a large number of aromatic sulfonic acids, and found many of these acids suitable as specific precipitants. These workers also developed a new method for the quantitative determination of amino acids, founded on their experimental observation that the amount of an amino acid salt precipitated from a solution was, at equilibrium, a function of the concentration of its ions in solution.

Methods most frequently employed for the estimation of individual amino acids in protein hydrolysates involve the selective precipitation of the respective amino acids in the form of salts, and in these methods the object is to precipitate as much of the salt as possible, 100% isolation being the ultimate goal. Unfortunately, most of the salts that are precipitated selectively are not precipitated quantitatively, but in the method of Bergmann, advantage is taken of such incomplete precipitation. For example, if an acidic reagent, RH, forms, with an amino acid, A, the binary salt, AH·R, it is found that in many cases the so-called solubility product of the participating ions is approximately a constant.

$$[AH^+] \times [R^-] = K \qquad (1)$$

Now let us assume that we obtain a precipitate with an amount of reagent r', which will contain equivalent amounts x, of the reagent, and y, of the amino acid; the amounts x' of the reagent and y' of the amino acid remaining in solution. Similarly using an amount of reagent r", the amounts x" and y" remain in solution, and it follows from equation (1) that

$$x' \cdot y' = x'' \cdot y'' = x''' \cdot y''' = \text{etc.} \qquad (2)$$

Equation (2) has been found to be generally valid, and if this is the case,

the amount Y of an amino acid can be determined in a solution of unknown content, for equation (2) can be written thus:

$$x'(Y-y_1) = x''(Y-y_2) = x'''(Y-y_3) \qquad (3)$$

All of the values in equation (3), with the exception of Y may be determined experimentally, and therefore Y may be calculated with a high degree of accuracy if equation (2) holds true for the particular experiment.

It was with the idea in mind of further developing these techniques of Bergmann, work which was interrupted by his untimely death, that a number of representative sulfonic acids were prepared. Two of these acids, naphthalene-β-sulfonic acid and 2-bromotoluene-5-sulfonic acid, were subsequently used for the purification of naturally occurring L(-) leucine.

The problem of the purification of L(-) leucine is one that has long interested chemists. The leucine first isolated from protein hydrolysates by Proust (10) and Braconnot (11), as well as the preparations made by later investigators, was usually purified by recrystallization from water or aqueous ethanol until the specific rotation, or some other physical property, was constant and the percentages of carbon, hydrogen, and nitrogen were in close agreement with the theoretical values. However, it is well known that natural occurring leucine cannot be freed from certain amino acid impurities by this treatment and that its purity is not established, unequivocally, by means of these criteria.

Fischer (12), one of the first to study leucine extensively, noticed the presence of a sulfur containing impurity in all his preparations. He destroyed this contaminant by heating the leucine with lead oxide for 7 hours at 165°, but the method was unsatisfactory as extensive racemization occurred. It was not generally recognized until around 1930 that this impurity was

methionine, or that it was present to any great extent. However, the gravity of the situation was revealed in 1935 by Mueller (13), who analyzed several well known commercial preparations of supposedly very high purity. In these methionine was present to the extent of 9.2%, 7.5%, and 2.7%.

In 1901 Fischer (14) reported the presence of another amino acid contaminant in leucine preparations obtained by the ester distillation method from the 80-85° fraction. The specific rotation of these crystalline fractions in 6N HCl at 20° with sodium light varied from about +22° to +23°. It is probable that this impurity was the isomeric isoleucine, since under these conditions it has a specific rotation of about + 40, while that of leucine is approximately +15°. Isoleucine was isolated a few years later by Ehrlich, who separated it from leucine by extraction of the copper salts with methyl alcohol; fractional crystallization failing in every case.

Other amino acid contaminants are undoubtedly present in commercial leucine preparations to a lesser extent, but these for the most part, are difficult to demonstrate qualitatively. Tyrosine can be tested for though, and this contaminant can be shown to be present in traces after rigorous fractional crystallization of the leucine.

The inadequacy of simple crystallization procedures in the purification of leucine was clearly shown by the experiments of Cohn et al (15), who worked with Hoffmann-La Roche and Eastman Kodak products. These preparations were recrystallized once from 50% alcohol, and twice from water, the most soluble and most insoluble fractions being discarded. Extensive solubility determinations were then made on the recrystallized leucine and also on the precipitates that remained after equilibrium was obtained. Large differences in the solubilities of the two products were observed, as well as a

still significant variance in the different determinations of each product. The specific rotations in 6N HCl were also greatly different in the two products.

According to Fox (16), attempts to purify natural leucine by esterification and fractional distillation, electrodialysis, precipitation of methionine as a mercury complex, and crystallization from acidified butyl alcohol have also proved unsuccessful.

In 1936, however, Fox (16) was able to obtain an L(-) leucine preparation of high analytical purity. Mixtures of leucine samples containing 2.6, 5.5, and 8.0% methionine were formylated by the methods of Fischer and Warburg (17) and Steiger (18). The formyl-leucine thus obtained was recrystallized 6 times from water, and then decomposed by refluxing with 10% HBr. The excess acid was removed by evaporation, the residue dissolved in water, and the evaporation repeated. The residue was once again dissolved in water, and the solvent removed by evaporation. This final residue was dissolved in a little water, neutralized with conc. NH_4OH, chilled, and the crystals filtered off. The leucine was then washed with alcohol until halide free, and finally recrystallized from water.

The yield of purified leucine that Fox obtained was 50% of theory, and it was stated to be sulfur free, ash free, and to have a specific rotation checking that of L(-) leucine obtained by synthesis and resolution. No supporting analytical data was given in the paper, but the work was repeated a few years later in Dunn's laboratory, and the leucine found to contain less than 0.004% methionine (19).

Continuing with the problem, Stoddard and Dunn (19) adopted the procedure of recrystallization of the leucine monohydrochloride, having first

tried the dileucine monohydrochloride, which failed to appreciably remove the sulfur. They used a technical grade leucine of fairly high purity, and fractionally crystallized the monohydrochloride several times. The salt was then dissolved in boiling water, the solution neutralized with NH_4OH, and the leucine recovered and washed with absolute ethanol. It was then recrystallized from water, again washed with absolute alcohol, and dried at 50°. The recovery of purified leucine was about 15% of the original material used.

This leucine preparation was then carefully analyzed for impurities with the following results being obtained:

moisture : 0.00 - 0.02%

ash : 0.00 - 0.01%

inorganic impurities:

NH_4^+
Fe^{++}
Fe^{+++}
PO_4^{---}
heavy metals

could not be demonstrated

Cl^- 0.004%

methionine: 0.10 - 0.05%

The equivalent weight, determined by formol titration with a glass electrode, was found to be 99.7 ± 0.08% of the theoretical value.

This preparation was undoubtedly of very high purity, but the methionine content leaves something to be desired, and there can be no assurance that the isoleucine was quantitatively removed. Isoleucine is undoubtedly present to a large extent in all commercial leucine preparations, and it is often difficult to detect as there are no qualitative tests available as yet,

and in solubility experiments it may escape dissolution, having the tendency to form mixed crystals with leucine as well as with methionine and other amino acids.

Fischer and Bergell (20) in 1902 suggested the introduction of the naphthalene-β-sulfonyl residue into the α-amino group as a means of transforming the amino acids into derivatives sparingly soluble in water, and subsequently this method was frequently employed for the identification of amino acids. Bergmann and Stein (7), in 1939, experimented further with naphthalene-β-sulfonic acid, and observed that it formed sparingly soluble salts with leucine, phenylalanine, arginine, histidine, tryptophane, methionine, and cysteine. On the basis of their results, these workers developed a procedure for obtaining highly purified L(-) leucine.

This procedure of Bergmann and Stein was used by the author as the starting point for the research on the development of a method to obtain naturally occurring L(-) leucine in large quantities and of a degree of purity that is believed to be the highest thus far obtained. This work will be described in detail in subsequent pages.

Syntheses

2,5-Dibromobenzenesulfonic acid:

The procedure first used was essentially that given by Bergmann et al.(9). To 5 grams of powdered p-dibromobenzene 10 cc of fuming H_2SO_4 (30% SO_3) were slowly added, and the reaction mixture heated on a steam bath for several hours. The mixture was then poured into 50 cc of ice, heated to 90°, and filtered while hot. The filtrate was then cooled to 0°, the acid which separated was recovered by filtration and recrystallized from water by the addition of conc. HCl.

This procedure was modified, however, as it was found that very little p-dibromobenzene appeared to react, and the yields were consequently very low. A steam jacketed bottle with a mechanical stirrer was adopted as the reaction chamber, and the quantity and strength of H_2SO_4 varied with the experiments.

By using 2 cc. of a mixture of 2/3 30% fuming H_2SO_4 and 1/3 60% fuming H_2SO_4 for every gram of p-dibromobenzene, and heating at 100° for approximately 10 hours, a 50% yield of the sulfonic acid was obtained. The recrystallized 2,5-dibromobenzene sulfonic acid melted at 111-112° C. The yields were lower both with acid of lower SO_3 content and of higher SO_3 content; the reactivity being low in the former case, and the amount of tarry by-products high in the latter. Too long heating also decreased the yield of the desired sulfonic acid due to the formation of bye products.

p-Hydroxyazobenzene-p'-sulfonic acid:

One mole of sulfanilic acid was dissolved in $2\frac{1}{2}$ liters of 3% Na_2CO_3, and the solution cooled to 0° C. To this was added a cold solution of 1 mole

of sodium nitrite dissolved in 500 cc. of water, followed by the addition, with stirring, of 1 1/5 moles of conc. HCl. This diazotization mixture was then slowly poured, with constant stirring, into a cold aqueous solution of 1 mole of phenol, and the mixture then made alkaline to litmus by the addition of 20% NaOH.

After standing for several hours in the cold room, the orange precipitate was recovered by filtration, and placed on porous plates to dry. This precipitate was then recrystallized three to four times from water to which conc. HCl had been added, deep red crystals being obtained in about 70% yield. The decomposition point of this sulfonic acid was approximately 250°.

The large number of recrystallizations are necessary as the sulfonic acid first precipitates mainly as the sodium salt, this being much less soluble than the free acid, and mixtures of the two are still obtained after only one or two recrystallizations.

2,6-Diiodophenol-4-sulfonic acid:

Phenol-4-sulfonic acid was first prepared by adding 70 cc. of 36 N H_2SO_4 to 100 gms. of phenol. This mixture was heated at 100° for 1 hour, and then poured into 100 cc. of water. One hundred cc. of 15 M NH_4OH were then added, and the mixture allowed to stand overnight. The precipitated sulfonic acid was then collected by filtration.

Next 73 gms. of the recrystallized phenol-4-sulfonic acid were dissolved in the minimum amount of water, and 150 cc. of 15 N NH_4OH added. To this was added, with shaking, a solution that had been made by dissolving 210 gms. of KI and 175 gms. I_2 in the minimum amount of water and then diluting to 1000 cc. Extreme caution was taken to keep all the reactants moist so that

no nitrogen triiodide would dry out on the walls of the container.

When the reaction mixture remained permanently colored by the further addition of the KI-I_2 solution, the reaction was deemed to be complete, and $NaHS\Theta_3$ was added to destroy the excess I_2. The solution was then acidified with conc. HCl, and the precipitate which formed recrystallized from water. The recrystallized acid was obtained in 70% yield, and had a decomposition point of approximately 295°.

2-Bromotoluene-5-sulfonic acid:

This sulfonic acid was prepared by slowly adding, with constant stirring, 250 gms. of O-bromotoluence to a mixture of 250 cc of 36 N $H_2S\Theta_4$ and 250 cc of 30% fuming $H_2S\Theta_4$. The temperature was maintained at about 45° until separate phases were no longer apparent, and then the sulfonation mixture was poured into a 4 liter beaker of ice.

After 10 hours in the cold room no precipitation had occurred, so the mixture was heated and 110 gms. of NaCl added. Upon recooling the sodium salt separated, was filtered off, and dried over KOH. The salt was converted to the free acid by dissolving in 2 liters of hot water and adding approximately 350 cc. of conc. HCl. The yield was 55% of theory, and the acid decomposed upon heating at about 320° C.

Naphthalene-β-sulfonic acid:

A three-necked flask containing 125 gms. of naphthalene was heated to 160°, and 115 cc. of 32 N $H_2S\Theta_4$ added dropwise over a period of 7-8 minutes. The temperature was maintained at 160° for 4 minutes after the addition of the $H_2S\Theta_4$, and then the sulfonation mixture poured carefully into 200 cc. of $H_2\Theta$.

Since 15% naphthalene-α-sulfonic acid was assumed to be formed by

this procedure, the mixture was boiled for 15 minutes to hydrolyze this isomer, and then steam distilled to remove the naphthalene that remained. The residue from the distillation was then treated with "Norit", filtered, and placed in the cold room.

After 24 hours the precipitate was collected, and placed in a vacuum dessicator over KOH. An additional crop of crystals was obtained by adding conc. HCl to the mother liquor. It was necessary to perform these operations in the cold room as the sulfonic acid is very hygroscopic, and also dissolves readily in its own mother liquor during filtration. Yields of 65-75% of theory were obtained by this method, and the melting point of the recrystallized acid was 104-106°. When crystallized from water-HCl, naphthalene-β-sulfonic acid contains water of hydration, and consequently the melting point will vary somewhat with the conditions employed.

Discussion:

The aromatic sulfonic acids are most readily obtained by the direct replacement of hydrogen by the sulfo group, and for this purpose a large number of sulfonating agents may be employed. Besides sulfuric acid of various concentrations and sulfur trioxide content and in the presence or absence of catalysts, such reagents as fluorosulfonic acid, chlorosulfonic acid, salts of chlorosulfonic acid, chlorosulfonic anhydride, and sulfamic acid may also be used. The choice of the reagent depends upon the compound to be sulfonated and the number of sulfo groups to be introduced. If fluoro- or chlorosulfonic acid is employed, the sulfonyl halide is often isolated first by the use of an excess of the sulfonating agent.

$$C_6H_6 + ClSO_3H \longrightarrow C_6H_5SO_3H + HCl$$

$$C_6H_5SO_3H + ClSO_3H \longrightarrow C_6H_5So_2Cl + H_2SO_4$$

In many cases side reactions may be of considerable importance, and conditions must be carefully chosen to reduce these to a minimum. Sulfone formation occurs to a varying extent in all sulfonations, and is especially prevalent when very reactive reagents such as sulfur trioxide or chlorosulfonic acid are employed. A number of substituted phenols may be converted by vigorous sulfonation into sulfonylides, bimolecular compounds containing two ester linkages, and bromine and iodine substituted compounds often undergo rearrangement or disproportionation. Also certain polyalkylated benzenes may undergo the Jacobsen reaction, and nitro substituted benzenes may react with explosive violence.

One cannot predict with certainty a priori in what position sulfonation will occur as the directive influence of certain substituents present in the aromatic ring is different in sulfonations than in other substitution reactions. For example, the halobenzenes sulfonate 100% para to the halogen while nitration occurs both ortho and para. In many cases, a change in the temperature at which the sulfonation is carried out will alter the position of the entering group or will produce a rearrangement of the primary reaction product into a more stable isomer. This is especially true in the naphthalene series, and in the case of the monosulfonation of naphthalene, the α-isomer is mainly formed at low temperatures, while the more stable β-isomer is prevalent at higher temperatures.

When sulfuric acid is employed as the sulfonating agent, the reaction is a reversible process, the theory of which is not too well understood. This process is complicated by the combination of the water formed in the reaction with sulfuric acid or the sulfonic acid to yield a hydrate. Also the concentration of a given aromatic compound in the sulfonation mixture not only varies

with the temperature but also with the quantity of water and of sulfonic acid present. Since there is no evidence that sulfuric acid ever adds to an olefin bond as hydroxyl and sulfonate groups, the old hypothesis that sulfonation proceeds through such an intermediate has no experimental foundation. In the case where the reagent is sulfur trioxide or chlorosulfonic acid, the intermediate formation of an addition product is more reasonable.

With the exception of aminosulfonic acids and certain other sulfonic acids of high molecular weight it is usually more convenient to isolate salts of the acids rather than the free acids because the acids are for the most part hygroscopic liquids or solids that are difficult to purify. Alkali salts are usually used for this purpose, and the sulfonates so obtained are generally readily soluble but less soluble than the corresponding acids. The alkali earth and lead salts, while usually soluble in water, frequently show marked variations for isomeric compounds so that it may be possible to separate sulfonation mixtures into their components by fractional crystallization of the calcium, barium, or lead salts of the sulfonic acids.

Purification of Naturally Occurring L(-) Leucine

Experimental Methods

The results of the following physical and chemical tests were used as criteria of purity, and also to follow the course of a purification procedure.

Melting Points:

Melting points were determined in capillary tubes heated in a copper block. The results were only recorded in the first series of experiments where it was desired to determine the number of recrystallizations necessary to give a constant value. The sensitivity of such a determination was of course low, but the values were useful as a check on the more accurate and sensitive tests. No determination was made of the decomposition point of the purified leucine, as such a value would be of practically no significance, the temperature range of decomposition being wide and subject to many variables. However, the salt of leucine and naphthalene-β-sulfonic acid has a fairly sharp melting point that can easily be reproduced.

Optical Rotations:

Since all of the naturally occurring amino acids, with the exception of glycine, have at least one asymmetric carbon atom, it was felt that the determination of the specific rotation would be an excellent method of following the course of a purification. Also since isoleucine has two asymmetric carbon atoms, and consequently a large specific rotation, it was believed that a very noticeable lowering of the readings would be observed with the removal of this impurity.

A Winkel-Zeiss polarimeter, able to hold tubes up to 2 dcm. in length, was used to determine these rotations. The light source was a sodium lamp. One decimeter tubes were used in the first series of determinations, but were abandoned due to the greater accuracy obtainable with two decimeter tubes. In cases where it was desired to control the temperature or to determine the change of rotation with temperature, a water-jacketed tube was used.

In the first series of determinations of the rotation of the leucine sulfonate, absolute ethanol was used as solvent, but the values obtained were not consistent due to evaporation of the solvent. Consequently methyl cellosolve was chosen for the remainder of the determinations. All the values on the leucine itself were obtained with 6.02 N HCl as solvent.

Absorption Spectra:

The ultra violet absorption spectrum of an aqueous solution of purified leucine was obtained by means of a Beckman Quartz Spectrophotometer. No determinations were made on the leucine sulfonate, as it was felt that other tests would be equally useful for the detection of impurities.

Solubility:

For the solubility determinations of the purified leucine, approximately 4 gms. of material were placed in each of three 125 cc. glass-stoppered bottles, and 60 cc. of redistilled water added. The bottles were then placed in a constant temperature water-bath set at 25.05 $\pm$ .05° C, and the bottles attached in such a manner that they would be agitated by the water current caused by a mechanical stirer.

After a period of 24 hours, one of the bottles was removed and

placed in a 55° C bath for three hours. It was then returned to the 25° C bath, thus allowing equilibrium to be approached from the saturated side.

For each solubility determination, approximately four cc. of the solution was withdrawn by pipette, and filtered into a previously weighed weighing bottle. The bottle was then reweighed and placed in a 100° C drying oven for 24 hours - this being the time required for complete drying. A third weighing was made, and the solubility thus calculated from these differences in weight. The first aliquot was withdrawn after 23 days, and the last after 29 days. At no time was crystallization observed during transfer of solution from the solubility bottle to the weighing bottle.

Following the first series of determinations, the undissolved leucine was recovered and dried. Approximately 3 gms. of this leucine was again placed in each of two of the solubility bottles, and 50 cc. of redistilled water added. The bottles were then placed in a mechanical shaker for 18 hours at approximately 23° C. One bottle was next placed in a 55° C bath for three hours, following which both bottles were placed in the 25° C bath. Individual solubility determinations were made as before, with the exception that the first aliquot was withdrawn after 5 days and the last after 11 days.

Amino Acid Nitrogen:

Amino acid nitrogen analyses were made by Mr. J. P. Cunningham using the Van Slyke manometric apparatus (21,22,23).

Tyrosine Analysis:

The tyrosine content of a given preparation was determined quantitatively with the Folin-Ciocalteu reagent and a photoelectric colorimeter (24).

This test has a sensitivity of one part in a million. Thanks are due Mr. D. J. Rice for carrying out these analyses.

Sulfur Analysis:

Qualitative micro sulfur analyses were made using the Zn-CaO fusion technique (25). In this method the sample is first fused with sulfur free Zn-CaO, perchloric acid added to the fusion mixture, and sulfide then detected with lead acetate. Such a method can detect as little as 2-3 γ of sulfur. Sodium fusion sulfur analyses were also made, but the results were of little value due to the lack of sensitivity of such a test.

Potentiometric Bromine Titration:

To determine the amount of easily oxidizable impurities present in a given preparation, 50 cc. solutions containing from .04-.06 gms. of material were titrated potentiometrically with bromine. The apparatus contained two sets of platinum electrodes, one set generating Br_2 from KBr at a known rate, and the other set indicating the point at which the Br_2 was no longer reduced by the material present in the solution (26,27). This method has a sensitivity of 10^{-7} equivalent of Br_2.

Such a potentiometric titration was mainly of value in detecting the presence of sulfur containing amino acids, especially methionine. Methionine is one of the major impurities in all leucine preparations, and is readily oxidizable to the corresponding sulfoxide by treatment with bromine (28,29). The benzene ring of the aromatic amino acids will also be brominated to a limited extent. When titrating the leucine salts of naphthalene-β-sulfonic acid, a correction had to be applied, as the naphthalene ring was also brominated under the conditions of the titration (30). Such a

correction factor was readily determined by running a blank on naphthalene-β-sulfonic acid itself.

Purification Procedures and Experimental Data

The procedure first used was essentially that given by Bergmann and Stein (7). As starting material, naturally occurring L(-) leucine (Lemke Co.) was employed, the probable leucine content being not over 70%.

To a hot solution of 10 gms. of leucine in 82.5 cc. of 1 N HCl was added a hot solution of 18.6 gms. of naphthalene-β-sulfonic acid in 17.5 cc. of water. This mixture was allowed to cool at 4° C for 24 hours, and the leucine sulfonate (or nasylate, as Bergmann recommended calling this salt) then filtered off and dried over KOH in vacuo. The yield of nasylate was 22.4 gms., or 86% of theory. The impure nasylate was then recrystallized five times from water solutions of concentrations from 10-20%, the recoveries being between 84 and 90%. The same procedure was repeated with another 10 gms. of leucine, but this time the nasylate was recrystallized from water solutions of concentrations from 5-10%. The recoveries in this case were somewhat less than before.

Determinations of the melting points, tyrosine content, and of the percent oxidizable by bromine were made following each crystallization in the first series, and of the melting points in the second series. Optical rotations were also taken, but the values were highly inaccurate due to evaporation of the solvent. These data are recorded in Table I.

Table I

	No. of Crystallizations	% Tyrosine	% Bromine Oxidizable	Melting Point °C
	1	0.5	6.9	180-183.5
	2	0.1	2.6	184-186
	3	0	0.31	186.5-188.5
Series I	4	0	0.10	186.5-188.5
	5	0	0.06	187-188
	6	0	0.04	187-188
	1	--	--	181-186
	2	--	--	185-187
	3	--	--	186-188.5
Series II	4	--	--	186.5-188
	5	--	--	186.5-188
	6	--	0.03	187-188
	Original Leucine	1.0	17.3	

The above results seem to indicate that after two recrystallizations of the nasylate the major proportion of the impurities is removed. However, after the fifth recrystallization there is still a significant amount of oxidizable impurities present.

After these first two series of crystallizations, it was decided that the nasylate was originally precipitated from too concentrated a solution to obtain good crystals and a satisfactory fractionation. Consequently a series of experiments was set up to determine the best ratio of solvent to reactants. The quantities of reagents used are shown in Table II.

Table II

Experiment Number	*	Wt.(gms) Leucine	Vol.(cc.) 1 N HCl	Wt (gms) Sulfonic acid	Vol.(cc) H_2O
1	2	1	16.5	1.86	3.5
2	3	1	24.75	1.86	5.25
3	4	1	33.0	1.86	7.0
4	5	1	41.25	1.86	8.75
5	8	1	66.0	1.86	14.0
6	16	1	132.0	1.86	28.0

* This column indicates the increase in proportion of solvent over that originally used.

It was found that the solutions remained too concentrated until five times the original amount of solvent was used. If the reactants were diluted much beyond this point, the yields were too low, and consequently it was decided that the proportions given in Experiment 4, Table II, would be the most satisfactory for future purifications.

Next 50 gms. of leucine (Lemke Co.) were precipitated as the nasylate using the above stated proportions. This precipitate was then recrystallized four times from aqueous solutions of concentrations from 3-5%. The mother liquor was retained in each case, and concentrated to approximately 1/3 its original volume to obtain further precipitation. The melting points and specific rotations of crystals from each fraction are recorded in Table III. The rotations are for 3% solutions of nasylate in methyl cellosolve, the temperature varying between 26-28° C.

Table III

No. of Crystallizations	$[\alpha]_D$	Melting Point °C
1	13.52	184.5-186
2	13.32	184-187
3	13.38	185-186.5
4	13.32	186-187
5	13.33	186-187
1c*	13.92	182-184
2c	13.14	186-187
3c	13.12	186-187.5
4c	13.15	186-187.5
5c	13.23	186-188

* This series is for the material recovered from the mother liquor.

The above determinations were made to discover whether the precipitates or the mother liquor were richer in impurities, and it would appear that there is little difference after the first crystallization. The fact that the last four specific rotations in the second series are somewhat lower than those of the first series can probably be attributed to a difference in temperature, each series having been determined on different days. It was thus seen that the impurities tend to remain in solution, and there would be no advantage gained in recrystallizing the nasylate obtained from the mother liquor rather than the original precipitate. Bromine titration of the four times recrystallized nasylate indicated oxidizable impurities amounting to 0.03%.

Since 2-bromotoluene-5-sulfonic acid had also been recommended as a specific precipitant for leucine (9), it was decided to perform two simultaneous purifications, using in one case naphthalene-β-sulfonic acid as precipitant, and 2-bromotoluene-5-sulfonic acid in the other. Twenty-five gram samples of leucine were used in each case, and precipitated from solutions of comparable concentrations. The original precipitations were followed by three recrystallizations.

The results indicated that there was little difference in the extent to which impurities were removed in each crystallization, and that the amount of oxidizable impurity remaining after the final crystallization was approximately the same in both cases. The values found by bromine titration were .03-.04%. The optical rotations for the two series are given in Table IV. The solvent was methyl cellosolve, and the concentration of each solution was approximately 7%.

Table IV

Precipitate	Cryst. No.	$[\alpha]_D^{25-26^\circ}$
Naphthalene-β-sulfonic acid	1	13.55
"	2	13.38
"	3	13.39
"	4	13.15
2-bromotoluene-5-sulfonic acid	1	12.16
"	2	11.97
"	3	12.08
"	4	12.06

Since naphthalene-β-sulfonic acid is available commercially in large quantities; while the 2-bromotoluene-5-sulfonic acid must be prepared in the laboratory, it was decided to use only the former acid in future purifications.

To determine if there would be any advantage gained by first crystallizing the impure leucine from various solvents and under varying conditions, the following series of experiments were performed:

(A) To 1000 cc. of boiling water were added 50 gms. of impure leucine and a few gms. of "Norit". The mixture was stirred and heated over a period of 45 minutes, and then filtered while still hot. The filtrate was allowed to stand at room temperature for 2 days, and the precipitate, 1, that had formed collected by filtration. Yield 10 gms. The filtrate was then concentrated to 800 cc., and allowed to cool at 0° C for several hours. The precipitate, 2, was collected (3 gms.), and the mother liquor concentrated to 700 cc. After standing at room temperature for several hours, precipitate, 3, was collected (5 gms.), and the mother liquor concentrated to 550 cc. Again the precipitate, 4, was collected by filtration (5 gms.), and the filtrate concentrated to 400 cc. Precipitate, 5, (5 gms.) was recovered after several hours, and the mother liquor finally concentrated to 200 cc. This concentrate was placed in the cold room over night, and precipitate, 6, then recovered (8 gms.).

(B) Ten grams of impure leucine and 1 gm. of "Norit" were added to 200 cc. of boiling water. The solution was stirred to obtain a saturated solution, and then filtered while hot. The filtrate was allowed to cool to 50°, and the precipitate, 1, that had formed was collected. To the mother liquor were added 70 cc. of ethanol, and the solution placed in the cold room

for 24 hours. Precipitate, 2, was then collected by filtration and the filtrate concentrated to 25 cc. After cooling this concentrate for 12 hours, precipitate, 3, was collected.

(C) Five grams of impure leucine and .2 gms. of "Norit" were added to 100 cc of H_2O, and the mixture heated to 60° C with constant stirring. The several grams of undissolved leucine and "Norit" were removed by filtration, and the filtrate placed in the cold room. After 24 hours, the precipitate, 1, was collected, and the mother liquor then concentrated to 25 cc. and returned to the cold room. Precipitate, 2, was recovered after 12 hours.

(D) Five grams of impure leucine and .2 gms. of "Norit" were added to 100 cc of water, and the mixture heated to 60° C with constant stirring. The undissolved leucine and "Norit" were removed by filtration, the solution reheated to 60° C, and 15 cc. of ethanol added. When the solution had cooled to 35° C, the precipitate, 1, that had formed was collected, and the filtrate placed in the cold room. Precipitate, 2, was collected after 24 hours, and the mother liquor concentrated to 45 cc. and returned to the cold room. Twenty-four hours later precipitate, 3, was collected.

(E) Ten grams of impure leucine were placed in a glass stoppered bottle containing 100 cc. of water, and the bottle placed in a mechanical shaking device for 3 hours. Two days after removal of the bottle from the shaker, the undissolved leucine was recovered, dissolved in boiling water (5% solution) to which a little "Norit" was added, the solution filtered and placed in the cold room. After 24 hours the precipitate, 1, was collected by filtration.

The original 100 cc. solution was concentrated to 75 cc., and placed

in the cold room for 24 hours. Precipitate, 2, was then recovered.

(F) To 100 cc. of glacial acetic acid were added, with constant stirring, 11 gms. of impure leucine. The undissolved material was removed by filtration, and 30 cc. of ethanol added to the filtrate. Precipitate, 1, formed immediately and was recovered. The filtrate was then concentrated to 35 cc and placed in the cold room. After 24 hours precipitate, 2, was collected. Two and a half grams of precipitate, 2, were dissolved in 100 cc. of boiling water, the solution filtered, and the filtrate placed in the cold room. Precipitate, 3, was collected after 24 hours.

All of the precipitates from the above six experiments were titrated with bromine, and the results are shown graphically in Figure I. It can be seen that in no case were the oxidizable impurities removed to any appreciable extent, and, therefore, fractional crystallizations of leucine itself are of no value in purification procedures.

Since none of the purification procedures that had been tried up to this time had succeeded in removing the sulfur present quantitatively, it was decided to treat the impure leucine with Rainey Nickel. A method had been worked out by Mozingo (32) for the hydrogenolysis of sulfur compounds, and if such a method succeeded with the impure leucine, the methionine present would be converted mainly to α-aminobutyric acid, a readily soluble compound.

Twenty grams of impure leucine were dissolved in 400 cc. of boiling water, and several grams of "Norit" added. The solution was then filtered, and the filtrate placed on a steam bath. Four spoonfulls of Rainey Nickel were then added, and the mixture stirred and heated for 6 hours. The catalyst was removed by filtration, and the filtrate evaporated to dryness. Less than a gram of material remained after this evaporation of solvent.

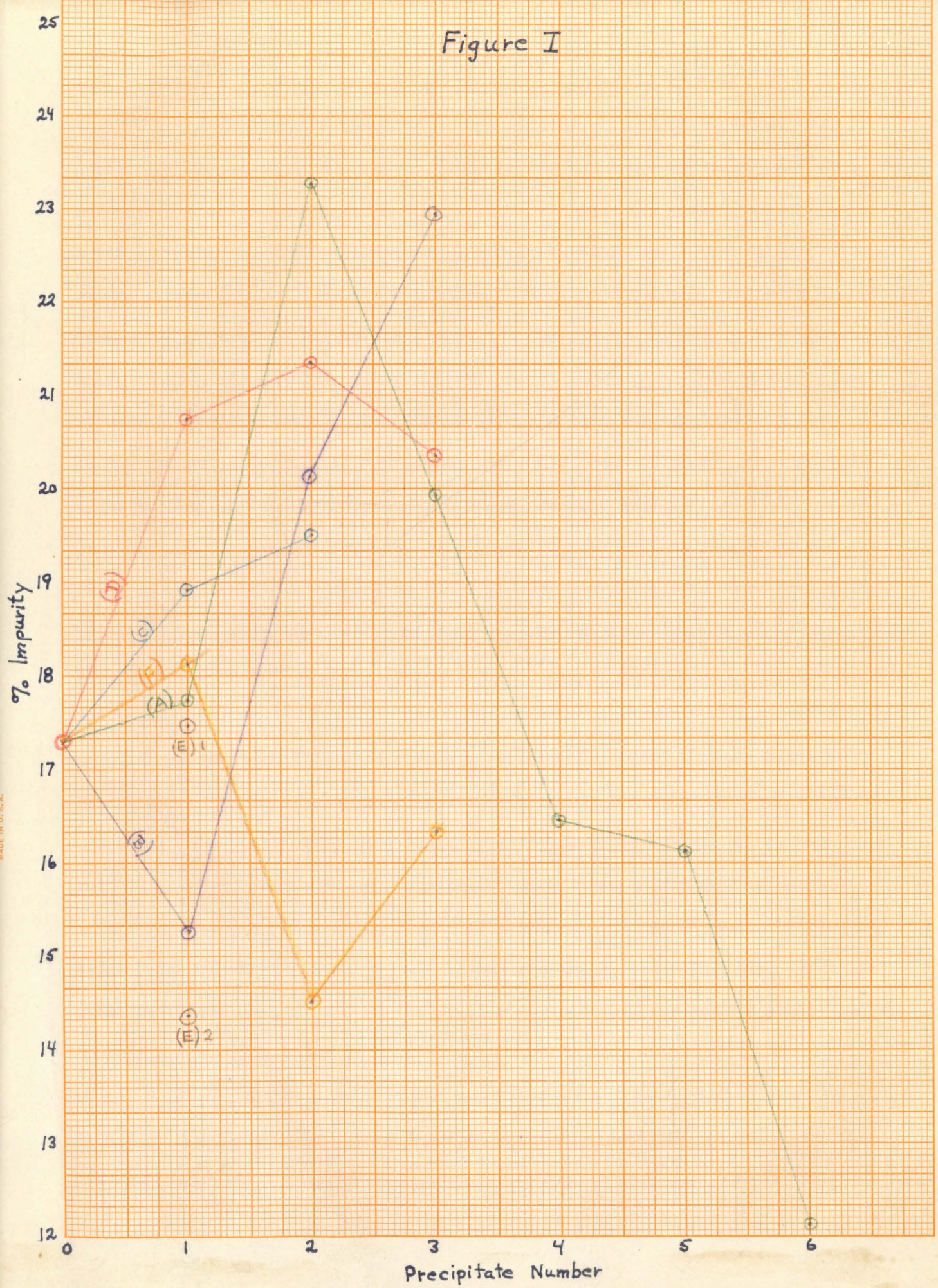
Figure I
% Impurity
Precipitate Number
25
24
23
22
21
20
19
18
17
16
15
14
13
12
0
1
2
3
4
5
6
(D)
(C)
(F)
(A)
(E) 1
(B)
(E) 2

The recovered Rainey Nickel was next added to 1100 cc. of boiling water, and the mixture stirred violently for 30 minutes. Again removal of the catalyst and evaporation of the filtrate yielded less than 1 gm. of material. The recovered Rainey Nickel was again added to 1100 cc. of hot water, but this time 25 cc. of acetic acid were added to bring the pH to 2-3. Once again removal of the Rainey Nickel and evaporation of the solvent yielded only 1-2 gms. of material. This material was colored green, and was very insoluble in boiling water. The leucine was evidently strongly held by the Rainey Nickel, and the little material recovered from the filtrates appeared to be present in the form of very insoluble metallic salts.

It was evident from the results of this experiment that many refinements in technique would have to be made before successful results could be obtained. It was not felt that further research into the use of Rainey Nickel was warranted at this time, and this phase of the problem was consequently abandoned.

A consideration of the reactions involved in the potentiometric titration of impure leucine made it seem worthwhile to carry out such a bromine oxidation as a preliminary step in the purification procedure. Since the sulfoxide of methionine is approximately twelve times as soluble as methionine itself (28,29) this sulfur containing impurity should be much more readily removed by fractional crystallization when present as the sulfoxide. Also the tyrosine present would be converted to the dibromo compound with a corresponding twofold increase in solubility.

Consequently a preliminary experiment was performed with a 15 gm. sample of impure leucine. This material was first dissolved in 620 cc. of warm 1 N HCl, and the solution passed through a column packed with "Norit." Bromine

water was added to the solution until a definite color persisted, and sodium bisulfite then added to remove this excess of bromine. The solution was heated to 70° C, and a solution of 30 gms. of naphthalene-β-sulfonic acid added to it. After chilling for 24 hours the precipitate, 1, was removed by filtration, and the filtrate concentrated to 120 cc. Precipitate, 1c, was recovered after 24 hours of cooling. Eighteen grams of precipitate, 1, were then recrystallized from 360 cc. of water, and precipitate, 2, collected.

In Table V are recorded the melting points, optical rotations in methyl cellosolve, tyrosine content, and percent of oxidizable impurity for each of the three precipitates. The bromine titrations were of course not too significant as the material had been previously oxidized, but they did indicate that further oxidation could take place. The Folin-Ciocalteu reagent was still useful for detecting tyrosine, as the dibromo derivative was found to give an even stronger color. Precipitate, 1c, would not dissolve in methyl cellosolve, and it was, therefore, impossible to determine the rotation in this solvent. Also the material discolored upon standing, so it would appear that it was considerably contaminated with impurities.

Table V

Ppt.	M.P.	$[\alpha]_D^{25-26^\circ}$	% Oxidized	Tyrosine
1	184-185	13.14	0	0
2	186.5-188	13.08	0	0
1c	d 210	--	.007	0

It was concluded from this preliminary experiment, that oxidation by bromine followed by precipitation and recrystallization of the nasylate

would be the most satisfactory purification procedure. Even if the oxidation did not aid in the complete removal of impurities, it would certainly not hinder such a purification. Thus it was decided to purify a large quantity of leucine (Lemke Co.) making use of such an oxidation followed by recrystallization of the nasylate as described previously.

Two hundred grams of impure leucine were dissolved in 2000 cc. of 1 N HCl, the solution heated to 70° C and passed through a column containing "Norit" to obtain a colorless solution. Bromine water was then added slowly and with constant stirring until a definite color persisted for a period of 20 minutes. This was followed by the addition of enough sodium bisulfite to completely remove the excess of bromine. The volume of the solution was then approximately 6000 cc.

Next, this leucine solution was heated to 70° C, and to it added 410 gms. of naphthalene-β-sulfonic acid monohydrate (Eastman Kodak) dissolved in 550 cc. of water. After standing for 24 hours at 4° C, the nasylate was recovered by filtration. Yield 451 gms. The nasylate was then recrystallized five times from water, the concentrations of the solutions being in each case 5%. The final yield of nasylate was 175 gms.

To recover the pure leucine, the nasylate was dissolved in 800 cc. of absolute ethanol and 145 cc. of redistilled pyridine added. The components were thoroughly mixed, and were then allowed to stand for 48 hours at room temperature. At the end of this time the leucine was collected on a filter, washed with absolute ethanol, and resuspended, while still wet, in 750 cc. of absolute ethanol. To this were added 110 cc. of pyridine, the mixture agitated for 30 minutes on a mechanical shaker, and again allowed to stand at room

temperature for 48 hours. The leucine was then recovered by filtration, washed with absolute ethanol and ether, and dried over KOH *in vacuo*. Yield 60 gms.

Finally, the leucine was dissolved in 1500 cc. of hot water and 700 cc. of absolute ethanol added. After 48 hours the leucine, which had crystallized as clusters of long rectangular plates, was recovered by filtration and dried over P_2O_5 *in vacuo*. Yield 37 gms. To insure the highest degree of purity, the leucine was recrystallized from 1000 cc. of water to which 500 cc. of absolute ethanol had been added. Yield 24 gms.

In Table VI are given the melting points, optical rotations in methyl cellosolve, and tyrosine content of the nasylate after each crystallization. Although there was some variation in the rotations, the values all agree within the limits of error, the temperature having varied somewhat with each determination.

Table VI

Cryst. No.	M.P. °C	$[\alpha]_D^{25-27^\circ}$	Tyrosine
1	185-187.5	13.32	0
2	186-188	13.57	0
3	187-188	13.36	0
4	187-188	13.63	0
5	187-188.5	13.16	0

To ascertain the probable purity of the leucine preparation, determinations were made of the sulfur content, tyrosine content, bromine oxidizable impurities, amino acid nitrogen, optical rotation, solubility,

and absorption spectrum. The results of these determinations are given below.

Sulfur: Analysis using the Zn-CaO fusion technique indicated that there was no sulfur present in the preparation. The sodium fusion technique also gave a negative result.

Tyrosine: There was no tyrosine present as determined colorimetrically with the Folin-Ciocalteu Reagent.

Oxidizable Impurities: Potentiometric titration of the purified leucine with bromine indicated that no oxidizable impurities were present.

Amino Acid Nitrogen: The Van Slyke manometric technique indicated a nitrogen content of 10.60%, or 99.3% of theory.

Optical Rotation: A large number of determinations were made using 6.02 N HCl as solvent, and with solutions of leucine concentrations varying from 3-5%. A water-jacketed, 2 dcm. polarimeter tube was used in all cases, and the temperature varied with each determination. The temperature coefficient of rotation was found to be a linear function within the temperature range studied, the value being + .07°/degree C. As well as could be determined, the rotation was not dependant on the ratio of concentration of mineral acid to amino acid in the cases studied. Bergmann (9) stated that for serine the rotation was no longer dependant on mineral acid concentration after the limiting value of 1.2 mineral acid/1 amino acid was reached. In all cases studied the ratio was much larger than this.

In Figure II are plotted all of the values obtained from the many determinations. From the curve drawn, the value for the rotation at 25° C can

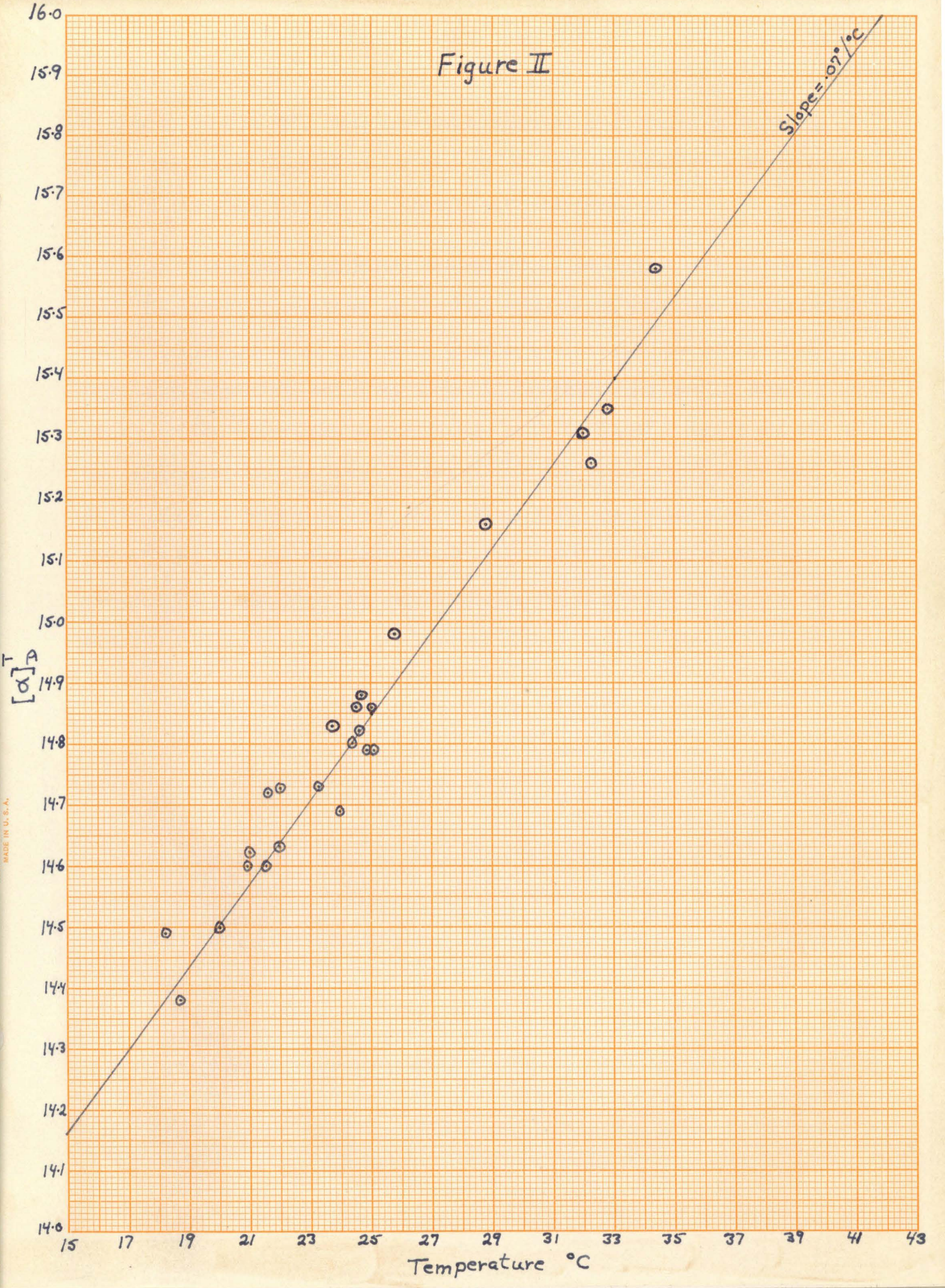
Figure II
Slope = .07°/°C
16.0
15.9
15.8
15.7
15.6
15.5
15.4
15.3
15.2
15.1
15.0
14.9
14.8
14.7
14.6
14.5
14.4
14.3
14.2
14.1
14.0
T A
[α]
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
Temperature °C

be seen to be + 14.85°. This same value can also be obtained by averaging all of the values obtained at, or near 25° C. It was permissable to average these values since they varied linearly with the temperature.

Solubility: Solubility determinations were first made on the purified leucine preparation, approaching equilibrium both from the saturated and undersaturated side. Following these determinations, the undissolved leucine was recovered, and a new series of determinations made on this material. The temperature was maintained at 25.05 ± .05° C during all of the determinations. In Table VII are plotted the values obtained in the first series of determinations, and in Table VIII, the values obtained with the material that remained undissolved after the first series of determinations. The solubilities are expressed as grams of leucine in 100 gms. of water.

Table VII

No. Days Equilibrated	Undersaturated Side	Undersaturated Side	Saturated Side
23	2.169		
24	2.159	2.160	
25	2.157 2.150	2.158 2.148	2.154
26	2.144		2.158 2.154
29	2.142 2.144	2.149 2.148	2.144 2.140

Mean value: S^{25° = 2.1516 gms./100 gms. water

Probable error: ± 0.006

Table VIII

No. Days Equilibrated	Undersaturated Side	Saturated Side
5	2.137	
7	2.161 2.141	2.140 2.158
8	2.128 2.168	2.136 2.162
11	2.152	2.143 2.132

Mean value: S^{25° = 2.1465 gms./100 gms. water

Probable error: ± 0.01

Absorption Spectrum: The ultra violet absorption spectrum of a 0.4% aqueous solution of the purified leucine is given in Figure III. As can be seen a smooth curve was obtained, with no peaks in the region of 260-270 mμ, where any aromatic amino acids would have absorbed if present.

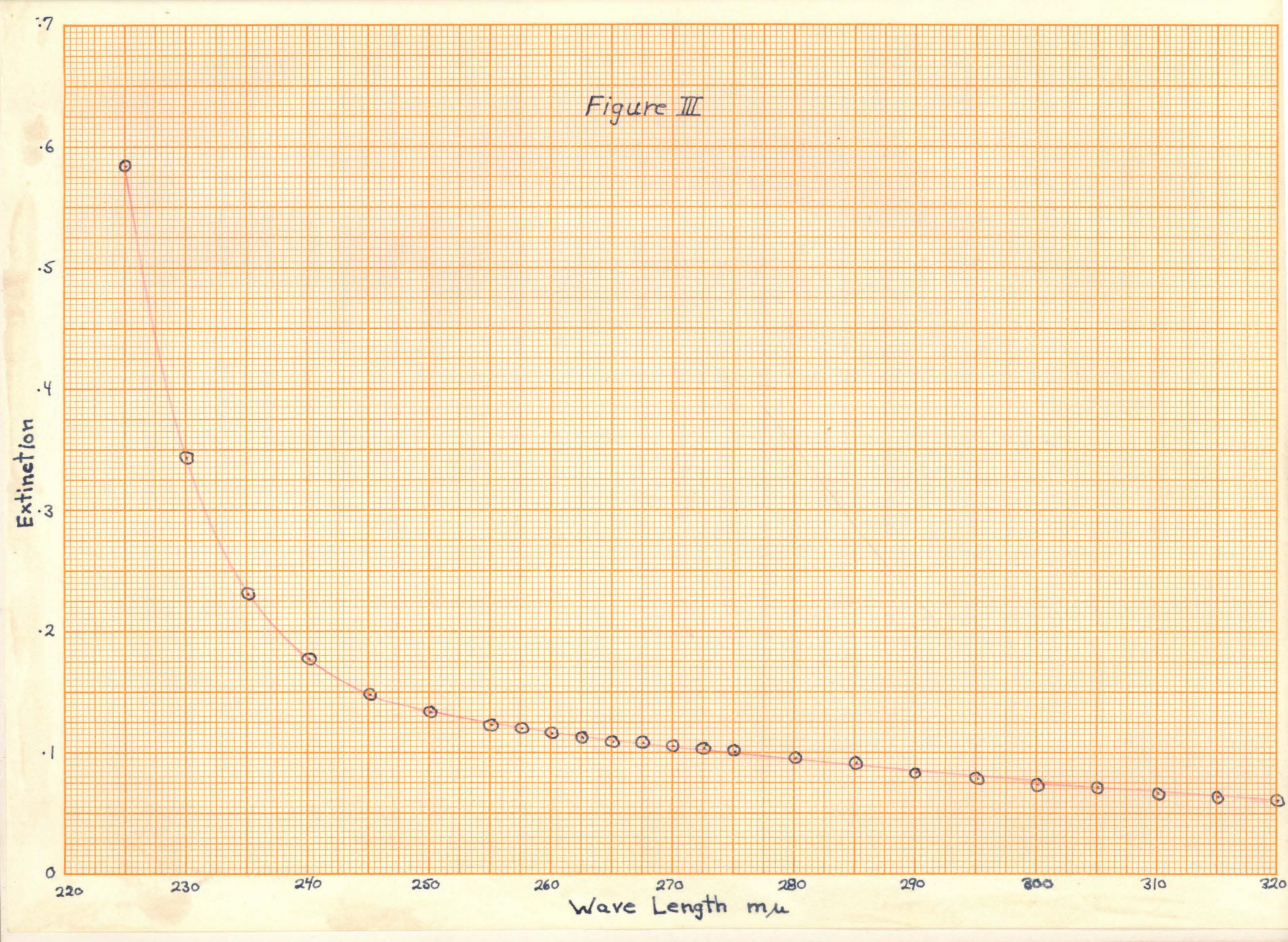
Figure III
.7
.6
.5
.4
.3
.2
.1
0
Extinction
220
230
240
250
260
270
280
290
300
310
320
Wave Length mμ

Discussion of Results

It is apparent from the results obtained that purification of naturally occurring L(-) leucine by preliminary bromine oxidation followed by recrystallization of the nasylate, yields a preparation of extremely high purity. The precipitations were effected from solutions of concentrations no greater than 5% to obtain the best crystals and most rapid purification. Even though the nasylate was always recrystallized four or five times, it is likely that two or three recrystallizations would be sufficient to obtain the same results.

One of the main requirements of a satisfactory purification procedure is that all of the sulfur containing impurities be removed. This requirement is satisfied by the procedure developed during this research. Other methods, that have previously been developed to obtain highly purified leucine, fail for the most part in this respect. Dunn's (19) preparation contained approximately 0.1 % methionine, Fox's (19) a small amount, but less than 0.004%, and Bergmann's preparation was stated to be sulfur free, but the method used to detect this impurity was not highly sensitive.

It must also be demonstrated that there are no other amino acids present to any large extent. With the exception of the aromatic amino acids, this is very difficult to do as there are no highly sensitive specific tests. However, the aromatic amino acids can be detected by bromine titration, and in the case of tyrosine, colorimetrically with the Folin-Ciocalteu Reagent.

Dunn made use of formol titrations as a criterion of purity, but such a method will fail to detect the presence of isoleucine, the most probable major impurity. Bergmann analyzed his preparation by means of his

quite accurate solubility method, and stated his preparation to be 97% pure. Amino acid nitrogen determinations also fail to distinguish between leucine and isoleucine, and it is, therefore, not at all unusual that theoretical or near theoretical values are readily obtained.

It is felt that solubility determinations, as described previously, are the most sensitive criteria of purity. Since the most probable contaminant, isoleucine, has a solubility twice as great as that of leucine at 25° C, the first series of solubility determinations will give higher values than the determinations on the undissolved residue, assuming that this amino acid is present.

For the purpose of estimating the purity of the leucine obtained by the author, we can assume that the solubilities of leucine and isoleucine are independant of each other, and also that isoleucine is the only contaminant present. Using Schmidt's value of 4.117 gms/100 gms. water for the solubility of isoleucine (32), and assuming that the value of 2.1465 gms/100 gms. water, obtained by the author in the second series of solubility determinations, is the true solubility of leucine, we see that there is a difference in solubility of 1.9705 gms/100 gms. water. Since the solubility of leucine was found to be 2.1516 gms/100 gms. water in the first series of determinations, this would indicate that isoleucine was present to the extent of 0.0051 parts in 1.9705. Or in other words the purity of the preparation was approximately 99.74%.

As further indication of the purity of the author's leucine preparation, we can compare the solubility obtained with the best values from the literature. Dunn (19) reported a value of 2.19 gms/100 gms. water at 25° C,

and Hlynka (33), using leucine purified by Fox (16), gave the value 2.20 gms/ 100 gms. water at 25° C. Thus, for the reasons discussed above, it would appear that the author's preparation was approximately 2.5% purer.

Similarly values for the optical rotation in 6 N HCl can be compared. Since isoleucine has a rotation of approximately 40° under comparable conditions, it will cause a rise in the rotation of leucine when present as an impurity. Dunn's best value for 25° C was 15.04° (34), and Bergmann (7) gave a value of 15.33° at 25° C. Once again it can be seen that the author's value, $[\alpha]_D^{25^\circ} = +14.85$ (3-5% solution in 6.02 N HCl), is considerably lower.

From a consideration of the results discussed above, it can be stated with a reasonable degree of certainty, that the procedure given herein for the purification of L(-) leucine is superior to any previously reported.

Summary

1. The following aromatic sulfonic acids were synthesized: 2,5-dibromobenzene sulfonic acid, ρ-hydroxyazobenzene-ρ'-sulfonic acid, 2,6-diiodophenol-4-sulfonic acid, 2-bromotoluene-5-sulfonic acid, and naphthalene-β-sulfonic acid. The last two sulfonic acids listed were used as specific precipitants for naturally occurring L(-) leucine.

2. A purification procedure was developed for L(-) leucine whereby the leucine was first oxidized by bromine, and then recrystallized four to five times as the salt of naphthalene-β-sulfonic acid. The leucine was recovered from the sulfonate by treatment of an alcoholic solution with pyridine, and then recrystallized twice from 33% aqueous ethanol.

3. Analyses of the purified leucine showed that sulfur, tyrosine, and all bromine oxidizable impurities were absent. The amino acid nitrogen content was found to be 99.3% of theory, and analysis using the solubility method indicated a purity of 99.7%.

4. The optical rotation was found to be, $[\alpha]_D^{25^\circ} = +14.85$ (3-5% in 6.02 N HCl), and have a temperature coefficient of $+0.07^\circ$/deg.C, between 18 and 34° C. This specific rotation was lower than any previously reported.

5. Solubility determinations were made at 25.05° C, first on the purified leucine, and then on the material that remained undissolved after equilibrium had been obtained. Equilibrium was approached both from the saturated and undersaturated sides. The mean value obtained was $2.149 \pm .008$

gms/100 gms. water. This value was several percent lower than any previously reported.

6. It was concluded that the purification procedure developed by the author, with the helpful guidance of Professor Carl Niemann, yielded a leucine preparation of greater purity than obtainable by any other procedure previously reported.

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Summary of the Thesis

by

Dudley W. Thomas

1. The following aromatic sulfonic acids were synthesized: 2,5-dibromobenzene sulfonic acid, p -hydroxyazobenzene- p' -sulfonic acid, 2,6-diiodophenol-4-sulfonic acid, 2-bromotoluene-5-sulfonic acid, and naphthalene-β -sulfonic acid. The last two sulfonic acids listed were used as specific precipitants for naturally occurring L(-) leucine.

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