

STUDIES ON THE SYNTHESIS OF DL-THREONINE
AND DL-HYDROXYPROLINE AND THE
CHARACTERIZATION AND ANALYSIS OF AMINO ACIDS
BY MEANS OF SOLUBILITY TEMPERATURES

By

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Thesis submitted to the Faculty of the Graduate School
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INTRODUCTION

Threonine, one of the four isomeric α -amino- β -hydroxy butyric acids, is one of the more difficult of the known essential amino acids to synthesize because of the difficulty of obtaining the desired isomer or its racemic mixture free of the other isomers. The initial object of this research was the study of new possible syntheses of DL-threonine and practical separations of DL-threonine DL-allothreonine mixtures. A necessary prerequisite to this undertaking was the development of an analytical method by which the composition of mixtures of the diastereoisomers could be determined. Attempts to analyze the mixtures by means of chromatography and partition chromatography were unsuccessful, but a solubility procedure was developed which proved satisfactory. The principles involved in the solubility analysis have also been shown to be useful for determining the purity or identity of other amino acids.

Once a method of analysis was available the catalytic reduction of compounds yielding derivatives of DL-threonine DL-allothreonine mixtures was begun. The reduction of ethyl α -acetamidoacetoacetate and ethyl O-methyl- α -oximinoacetoacetate was carried out over a wide variety of catalysts in the hope that DL-threonine might be formed preferentially with one of them. The α -propionamido- and

α -succinamido- acetoacetic esters were also examined, but much less extensively than the afore mentioned compounds. This hydrogenation study, together with the development of the solubility method of analysis, comprises the bulk of the work on the theonine problem.

In addition to the work related to DL-threonine, the synthesis of DL-hydroxyproline by the method of Feofilaktov and Onischenko (1) was studied. These authors give only sparse experimental directions for this difficult preparation. This work was undertaken to supply the Poultry Department's needs for synthetic DL-hydroxyproline.

THE SYNTHESIS OF THREONINE-ALLOTHREONINE MIXTURES

BY CATALYTIC HYDROGENATION

INTRODUCTION

At the time of the isolation of threonine from fibrin by McCoy, Meyer and Rose (2), two syntheses of α -amino- β -hydroxy-n-butyric acid had been reported in the literature, but the configuration of these products was unknown. Burch (3) synthesized a mixture of DL-threonine and DL-allothreonine by the ammonolysis of α -chloro- β -hydroxy-n-butyric acid, obtained by the addition of hypochlorous acid to crotonic acid. In addition to the desired α -amino acids, the product also contained the isomeric α -hydroxy- β -amino-n-butyric acids. Abderhalden and Heyns (4) prepared the compound by the addition of mercuric acetate in methanol to ethyl crotonate. The mercury addition compound on treatment with potassium bromide followed by bromine gave ethyl α -bromo- β -methoxy-n-butyrate. Careful alkaline hydrolysis of the ester, ammonolysis of the α -bromine, and cleavage of the methyl ether with concentrated hydrobromic acid led to the desired product. Only one of the possible racemates could be isolated from the product and its melting point indicates that it was allothreonine.

The thorough investigation of the problem by Carter and his coworkers (5,6,7) finally led to a satisfactory

laboratory preparation of DL-threonine (8). This synthesis is similar to that of Abderhalden and Heyns, but differs in that the starting material is crotonic acid instead of the ester. The two synthesis also differ after the α -amino- β -methoxy-n-butyric acids are obtained. The synthesis of Carter converts these compounds to the N-formyl derivatives, from which pure N-formyl-O-methyl-DL-threonine can be obtained by crystallization. Hydrolysis with hydrobromic acid then produces DL-threonine. The diastereoisomeric DL-allothreonine can be obtained from the mother liquors from the crystallization of the formyl derivatives by suitable treatment.

Adkins and Reeve (9) used acetoacetic ester to prepare a mixture of DL-threonine and DL-allothreonine. Nitrosation, followed by alkylation of the oxime with diethyl sulfate, yielded ethyl O-ethyl- α -oximinoacetoacetate. This was reduced with hydrogen and Raney nickel. Hydrolysis of the reduction product gave a 75% yield of mixed theonines. This synthesis is direct, but suffers the disadvantage of giving a mixture which cannot be readily separated into its pure components.

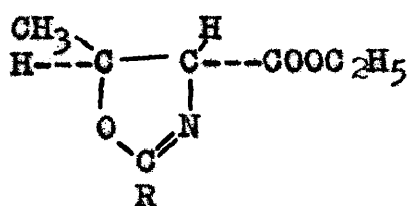
Several syntheses of DL-threonine have been reported quite recently. The novel synthesis of Birkofer (10) involved the reduction of α -diazoacetoacetic ester with platinum oxide in the presence of sulfuric acid. The required diazo compound was obtained by the reaction of acetyl chloride and diazoacetic ester. Hydrolysis

of the ester obtained by reduction gave a mixture of DL-threonine and DL-allothreonine in 40% yield.

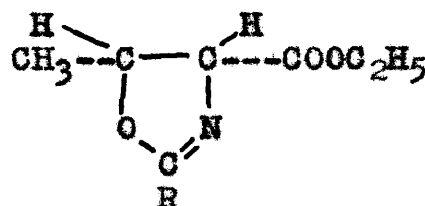
Pfister, et al., (11) have prepared threonine from the α -bromo- β -methoxy-n-butyric acid which is made by the addition of the elements of methyl hypobromite to crotonic acid. Amination of this acid, which has the allothreonine configuration, yielded nearly pure DL-allothreonine (12). However, if an amide of the acid, such as the piperidide, was aminated, rather clean inversion of the α -carbon was obtained. The overall yield of pure DL-threonine based on crotonic acid was 30%.

A second synthesis by Pfister, et al., (13) and the synthesis of Attenburrow, Elliot and Penny (14, 15) are closely related. Pfister reduced ethyl α -acetamidoacetate with platinum oxide to produce ethyl α -acetamido- β -hydroxy-n-butyrate, 80-85% of which had a configuration corresponding to that of allothreonine. Treatment of the ester with thionyl chloride gave mainly trans-2,5-dimethyl-4-carbethoxyoxazoline I ($R = CH_3$), which on hydrolysis with dilute hydrochloric acid yielded DL-threonine of better than 80% purity. Elliot prepared a mixture of the trans and cis 2-phenyl-5-methyl-4-carbethoxyoxazolines I and II ($R = C_6H_5$) by reduction of ethyl α -benzamidoacetate with Raney nickel and subsequent treatment with thionyl chloride. The mixture of isomers was refluxed with dilute aqueous base to convert the cis-oxazoline II to the more stable trans form I, with simultaneous hydrolysis of the ester occurring. Since the trans-oxazoline

has the threonine configuration, only hydrolysis with acid was necessary to produce DL-threonine.



I



II

The investigation of the hydrogenation of compounds such as ethyl α -acetamidoacetoacetate and ethyl O-methyl- α -oximinoacetoacetate over various catalysts was undertaken as a possible method of synthesis for DL-threonine. It was hoped that a catalyst might be found which would yield DL-threonine predominately, for such a procedure would be direct and applicable to large scale preparation of this amino acid. The reduction of N-methyl- α -aminopropiophenone hydrochloride with platinum oxide (16) serves as an example of the type of specificity which was sought. Although two diastereoisomeric forms of the product are possible in this case, only dl-ephedrine was obtained. The reduction of α -oximinoketones, such as α -oximinopropiophenone, with palladium on carbon catalyst in alcoholic hydrochloric acid has also been shown to yield only one of two possible diastereoisomers (17,18).

Throughout the remainder of this paper the capital letter prefix DL will be omitted, and the terms threonine and allothreonine will refer to the racemic amino acids.

DISCUSSION AND RESULTS

The reduction of ethyl α -acetamidoacetoacetate with platinum oxide and nickel catalysts has been reported to yield mainly allothreonine (13, 19). As a first step, the reported reductions with Adams platinum catalyst were reinvestigated. We were unable to duplicate the results of Albertson, et al., (19) who reduced the material in acetic acid, but reduction of the carbonyl was readily effected in water solution with hydrogen at atmospheric pressure as reported by Pfister, et al. (13). After hydrolysis, the isolated amino acid mixture analyzed for 86% allothreonine. This is in good agreement with the figure of 80-85% obtained by Pfister. The reduction with platinum oxide also proceeded slowly in absolute ethanol, the product again containing 86% allothreonine.

When the platinum catalyzed reduction in water solution was run at 40 atmospheres pressure, 79% allothreonine was found. The smaller amount of allothreonine in this sample was not due to a change in the ratio of threonine to allothreonine formed, but due to the presence of larger amounts of impurities. In order to minimize the effect of varying amounts of impurities in the samples, both threonine and allothreonine were determined. The results are best expressed as the percent threonine relative to the total amount of threonine and allothreonine present, and this figure will be given throughout the remainder of this discussion except where noted. In the case of

the hydrogenations in aqueous solution at one and 40 atmospheres pressure with platinum the relative amounts of threonine become 12% and 11% respectively. The use of platinum supported on active carbon as a catalyst yielded a mixture containing 20% threonine.

Palladium was next examined as a catalyst for the reduction of ethyl α -acetamidoacetoacetate. Palladium on carbon, palladium oxide, and colloidal palladium all failed to give any reduction in aqueous solution at atmospheric pressure. Palladium oxide in acetic acid at atmospheric pressure also caused no reduction. Reductions were carried out in a steel vessel at 40 atmospheres pressure, the temperature being raised slowly from 25° to 150°, and both water and alcohol were tried as solvents. Negligible amounts (1-9%) of amino acid were obtained after acid hydrolysis, and these were contaminated with large amounts of iron and halogen. It was felt that these samples were too impure to yield useful analyses. When the high pressure reductions were repeated using a glass liner, no amino acids could be isolated.

A ruthenium on carbon catalyst, prepared by the reduction of ruthenium chloride with hydrazine hydrate, gave no reduction at atmospheric pressure when the compound was dissolved in water or acetic acid. A yield of 9% of crude brown solid was obtained when the reduction was carried out at 40 atmospheres pressure in absolute ethanol. A very active ruthenium catalyst was formed by

reducing ruthenium chloride in the presence of carbon with hydrogen at 70 atmospheres pressure and a temperature of 100°. This catalyst rapidly effected the reduction of the acetamido compound in aqueous solution. The amino acids isolated in this case contained 32% threonine.

Rhodium, osmium and iridium on carbon catalysts all reduced ethyl α -acetamidoacetoacetate in aqueous solution at one atmosphere hydrogen pressure. The products isolated contained 46% threonine for rhodium and 33% threonine for both osmium and iridium. These three catalysts failed to give any reduction when acetic acid was the solvent. It is interesting to note that the rhodium catalyst was active only for a short time and required several reactivations by shaking with air to complete the reduction of 0.02 moles of the compound.

A series of Raney type catalysts were also examined for their effect on the amount of threonine produced by the reduction of α -acetamidoacetoacetic ester. Reductions with Raney nickel, copper and cobalt were run in alcohol solution at hydrogen pressures of 80-85 atmospheres. As would be expected, nickel was the most active of the three metals, reducing the carbonyl at 50°. Reduction with Raney cobalt started slowly at 70-80°, but became appreciable only after a temperature of 100° was reached. The reduction over Raney copper was run at 125°. The amino acid mixture isolated from the nickel reduction contained 24% threonine. Pfister, et al., (13) also reduced the

acetamido compound with nickel and their product contained 30%. Raney copper yielded allothreonine predominately, only 14% threonine being produced. Raney cobalt gave an amino acid mixture containing 62% threonine, which was the highest yield of the desired racemate in all the reductions that were run.

Iron-aluminum and vanadium-aluminum alloys were prepared, and after they were powdered, Raney type catalysts were made from them. Raney iron has been prepared by Paul and Hilly (20), who claimed that it would not catalyze the reduction of ethylenic compounds although acetylenes could be readily reduced to ethylenic compounds. Paul and Hilly treated the iron-aluminum alloy twice with sodium hydroxide solution at temperatures of 80-90°, and it was felt that a more active catalyst, possibly capable of reducing ketones, might be produced by milder treatment with alkali. There is no reference in the literature to a vanadium catalyst of the Raney type.

Neither the iron nor the vanadium gave any evidence of catalyzing the reduction of ethyl α -acetamidoacetate in ethanol solution up to temperatures of 250° and at hydrogen pressures of 100 atmospheres or more. The material removed from the reduction vessel after these trials, had a strong odor of ethyl acetate and contained appreciable amounts of ammonia or a volatile amine. After acid hydrolysis and removal of the volatile base, the only product that could be isolated was glycine, which

was identified in both cases by solubility measurements. The isolation of glycine and the presence of ammonia or an amine is evidence that excessive rupture of bonds occurred at the elevated temperatures used in these runs. It should be noted that hydrolysis with hydrochloric acid of the starting material yields aminoacetone hydrochloride.

In addition to the metal catalysts, copper chromite and zinc chromite catalysts were used to reduce the acetamido compound. Reduction with copper chromite proceeded readily at 125° in ethanol solution, giving a mixture containing 39% threonine. Zinc chromite would not effect the reduction at lower temperatures and when the temperature was raised to 250° there was extensive cleavage of the molecule as in the case of the Raney iron and vanadium catalysts. Glycine was the only amino acid which could be isolated from the reduction mixture.

A summary of these reductions of ethyl α -acetamidoacetoacetate and the analyses of the products isolated is given in Table I. Several additional reductions which will be discussed later are also included in this table.

In the catalytic hydrogenation of olefinic hydrocarbons on metallic surfaces there is evidence for the assumption that the chemisorption of the hydrocarbon is an important factor (21). Adsorption of the olefin is presumed to occur by opening of the double bond and attachment of the carbon atoms to two catalyst atoms. If a similar chemisorption takes place before the reduc-

TABLE I

SUMMARY OF THE CATALYTIC HYDROGENATIONS

Catalyst	Amount of Catalyst, g.	Moles, Compd.	Solvent, ml.	Press. atm.	Temp. °C	Time hr.
PtO ₂	0.3	0.10	100 H ₂ O	1	25	3
PtO ₂	0.3	0.10	100 EtOH	1	25	31
PtO ₂	0.3	0.10	100 H ₂ O	40	25	3
PtO ₂	0.2	0.10	" "	1	25	2
Pt (C)	1.0	0.02	50 "	1	25	4
Ru (C)	2.0	0.05	" "	1	25	1
Ru (C)	2.0	0.05	" "	1	25	2
Ru (C)	2.0	0.10	100 "	1	25	1
Ru (C)	1.5	0.05	" "	1	65	1
Ir (C)	1.5	0.02	50 "	1	25	3
Ir (C)	2.0	0.05	" "	40	25	1
Rh (C)	1.0	0.02	" "	1	25	9
Os (C)	1.0	0.02	" "	1	25	16
Cu Raney	2.0	0.10	150 EtOH	85	125	2
Ni Raney	2.0	0.10	" "	80	50	2
Co Raney	2.0	0.10	" "	85	100	1
CuCrBaO	2.0	0.10	" "	190	125	1
ZnCrO	2.0	0.10	" "	200	250	1
Fe Raney	2.0	0.10	" "	100	250	2
V Raney	2.0	0.10	" "	190	250	1

TABLE I (CONTINUED)

OF ETHYL α -ACETAMIDOACETOACETATE

% H ₂ Uptake	Yield, %	% Threonine	% Allo- threonine	Relative % Threonine	Neutral Equiv. (Calcd. 119)
102	79	12	86	12	120
93	73	--	86	--	121
65	42	10	79	11	118
50	40	11	83	12	123
90	42	19	78	20	120
102	80	30	65	32	118
103	86	32	65	33	121
50	30	28	65	30	122
88	66	34	63	35	120
108	67	31	62	33	121
60	59	29	60	33	119
120	50	44	51	46	123
110	67	32	65	33	121
83	55	13	80	14	121
158	85	23	74	24	120
128	82	63	38	62	122
170	85	38	60	39	120
42	--	36% of glycine, only product			78
21	--	33% of " , " " "			77
60	--	64% of " , " " "			77

tion of a carbonyl group is accomplished, it will be noted that in the case of ethyl α -acetamidoacetate, the adsorbed molecules can exist in diastereoisomeric forms corresponding to threonine and allothreonine.

~~Stewart~~ models of the diastereoisomeric adsorbed molecules were prepared and examined. For the model corresponding to allothreonine, it was found that the bonds of the open carbonyl group could squarely contact a plane surface with both the amide and ester linkages also lying flat on the surface. However, either the amide or ester group had to be some distance away from the surface for the free carbonyl bonds of the threonine model to be in good contact.

With this observation in mind, ethyl α -propionamidoacetate was prepared to see if the substitution of a methyl group for a hydrogen in the acetyl radical would increase the relative amount of threonine produced over a given catalyst. The additional methyl group should render the adsorption of the amide linkage on the catalyst more difficult from the steric point of view, all other factors remaining the same. Unfortunately, the added methyl group should at the same time increase the ease of polarization of the amide linkage so that its adsorption would be facilitated. Assuming that the adsorption of the amide linkage influences the course of reduction, then these two opposing effects would tend to counteract one another.

The α -propionamidoacetoacetic ester was reduced with platinum oxide, ruthenium on carbon and rhodium on carbon catalysts, at atmospheric pressure in aqueous solution. The amino acids isolated from these runs were analyzed in the same way as were the mixtures obtained from the acetamido compound. Pertinent data on these reductions are given in Table II.

TABLE II

REDUCTION OF ETHYL α -PROPIONAMIDOACETOACETATE*

Catalyst	Amt. Cat. g.	Time hr.	% H ₂ Uptake	Yield, %	Relative % of Threonine	Neutral Equiv.
PtO ₂	0.2	4	96	74	12	120
Ru(C)	2.0	1	100	80	34	119
Rh(C)	2.0	7	109	71	45	121

* 0.05 moles reduced in all runs.

Ethyl α -succinamidoacetoacetate was also prepared and was reduced over the same three catalysts in water solution in the form of its lithium salt. It was hoped that the carboxylate ion would tend to remain hydrated and not be adsorbed on the catalyst. If this were the case, adsorption of the amide group should not occur too readily. The amino acids isolated from these reductions were found to be contaminated with lithium succinate which had to be removed before analyses could be run. There is danger of altering the threonine-allo-threonine ratio during an operation of this type since

more of one diastereoisomer may be lost than of the other. In addition, the reductions of the succinamido compound did not proceed well. As a result of these two factors, it is felt that the analyses on the products for this compound are not as representative as are those on the acetamido and propionamido compounds. A summary of these reductions is given in Table III.

TABLE III

HYDROGENATION OF THE LITHIUM SALT OF ETHYL
 α -SUCCINAMIDOACETOACETATE*

Catalyst	Amt. Cat. g.	Time hr.	% H ₂ Uptake	Yield, %	Relative % of Threonine	Neutral Equiv.
PtO ₂	0.3	6	90	29	6	120
Ru(C)	2.0	29	87	34	23	135
Rh(C)	3.0	5	92	34	30	130

* 0.02 moles reduced in all runs.

For convenience, the relative amounts of threonine produced from the three acyl derivatives of α -amino-acetoacetic ester with platinum, rhodium and ruthenium catalysts are given in Table IV.

TABLE IV

RELATIVE AMOUNTS OF THREONINE FORMED BY THE REDUCTION OF
N-ACYL- α -AMINOACETOACETIC ESTERS

Catalyst	Acyl Group		
	Acetyl	Propionyl	Succinoyl
PtO ₂	12	12	6
Rh(C)	46	45	30
Ru(C)	32	34	23

The results for the acetyl and propionyl compounds are identical within the limits of experimental error. All of the figures for the succinoyl derivative are considerably lower than the corresponding analyses for the other two compounds. It is believed that this lowering is due to the loss of threonine incurred during the removal of the lithium succinate which was present in the samples as initially isolated. Since threonine is somewhat more soluble than allothreonine, its recovery would not be as complete as that of allothreonine. It appears that the relative amount of threonine produced is independent of the acyl substituent. Undoubtedly there are minor changes, but to be observed a more refined investigation would be necessary. Additional evidence of the constant amount of threonine produced by the reduction of N-acyl- α -aminoacetoacetic esters is furnished by the work of Attenburrow, Elliot and Penny (14) who reduced ethyl α -benzamidoacetoacetate with Raney nickel. Their product contained 25-30% threonine, while reduction of

the acetamido compound over nickel in this laboratory yielded 24% threonine.

The hydrogenation of ethyl O-methyl- α -oximinoacetate was attempted over most of the catalysts which had been used for the acetamido compound. This compound is difficult to reduce in good yield to the desired product, ethyl α -amino- β -hydroxy-n-butyrate, because of its tendency to form by-products, the most important of which is 2,5-dimethyl-3,6-dicarbethoxypyrazine (9). All reductions with the platinum metals were run in absolute ethanol as a solvent with a glass liner to prevent contamination of the products and possible poisoning of the catalysts.

The yield of amino acids was only 9% when platinum oxide was the catalyst, and this yield was not improved by increasing the pressure from 85 to 300 atmospheres. This product analyzed for 37% threonine. In addition to the amino acids, an unidentified water soluble oil was present which was not observed with any other catalyst.

Palladium on carbon failed to give any threonine, but considerable amounts of 2,5-dimethyl-3,6-dicarbethoxypyrazine were isolated. This material was identified by a mixed melting point with an authentic sample of the material.

Rhodium, osmium, ruthenium and iridium on carbon catalysts all gave yields of threonine-allothreonine mixtures of about 50%. Small amounts of pyrazine were found in the products from the rhodium and osmium

reductions. A small amount of water insoluble oil was present in the ruthenium reduction. The amount of threonine in the amino acid mixtures isolated from these four runs varied from 34-41%.

Raney nickel and cobalt satisfactorily catalyzed the reduction of the O-methyl- α -oximinooacetic ester, giving higher yields of mixed amino acids than any of the platinum metal catalysts. These reductions were run in ethanol at pressures of about 300 atmospheres. The product from the nickel hydrogenation contained 36% threonine, while that from cobalt contained 43%. Raney copper was ineffective against this compound. Although more than the theoretical amount of hydrogen was absorbed, no amino acids could be isolated and there was extensive decomposition of the material as evidenced by the presence of large amounts of brown water-insoluble oil and ammonia.

Copper chromite was used as a catalyst for the reduction, and as in the case of copper slightly more than the theoretical amount of hydrogen was absorbed. The temperature of the reduction was 135°. The reaction mixture was dark in color and contained considerable amounts of ammonia. When worked up in the usual way, about 4% of light brown solid was obtained. This crude material was not completely water soluble, and had a neutral equivalent of 84 as determined by a formol titration. This material was probably glycine which has a theoretical neutral equivalent of 75.

Table V summarizes these reductions of ethyl O-methyl- α -oximinoacetoacetate, and the compositions of the products isolated from them.

It was noted that a crude relationship seems to exist between the relative percent of threonine produced by a metal catalyst and the distance between the centers of the catalyst atoms for the reduction of ethyl α -acetamidoacetoacetate and ethyl O-methyl- α -oximinoacetoacetate. The metals platinum, rhodium, iridium, nickel and copper crystallize in the cubic closest-packed arrangement in which each atom is surrounded by twelve atoms equidistantly spaced. Osmium, ruthenium and cobalt, however, have the hexagonal closest-packed arrangement and each of their atoms is surrounded by twelve atoms, six at one distance and six at some greater distance. For the first group of metals no assumptions regarding the distances between atoms is necessary since there is only one dimension describing their lattice, but the hexagonal elements have adjacent atoms at two distances. For this latter group, the assumption was made that approximately half of the exposed adjacent atoms were at one distance and half at the other. On this basis, the average value of the two parameters was used for plotting the results for the three metals having the hexagonal structure.

The relative percentages of threonine produced by the reduction of the acetamido compound and the oximino

TABLE V

SUMMARY OF THE CATALYTIC HYDROGENATIONS

Catalyst	Amount of Catalyst g.	Moles, Compd.	Solvent, ml.	Press. atm.	Temp. °C	Time hr.
PtO ₂	0.3	0.10	50 EtOH	85	100	1
Rh(C)	3.0	0.10	" "	85	25	2
Os(C)	3.0	0.10	" "	85	50	1
Ru(C)	3.0	0.10	" "	85	60	1½
Ir(C)	3.0	0.10	" "	85	25	½
Ni Raney	2.0	0.20	100 "	300	90	½
Co Raney	2.0	0.10	125 "	270	130	½
CuCrBaO	2.0	0.15	150 "	250	135	2

TABLE V (CONTINUED)

OF ETHYL O-METHYL- α -OXIMINOACETOACETATE

% H ₂ Uptake	Yield, %	% Threonine	% Allo- threonine	Relative % Threonine	Neutral Equiv. (Calcd. 119)
73	9	34	59	37	120
123	50	38	54	41	120
119	45	33	64	34	120
114	40	34	61	36	120
108	61	34	64	35	121
94	75	35	62	36	118
130	63	39	51	43	120
104	4	--	Probably glycine	--	84

ether over metal catalysts, as well as the distances between centers for the metal atoms, are listed in Table VI, and these data are plotted in Figure 1.

An explanation of these relationships is not evident; however, it is felt that such a result is not surprising when the work of Beeck concerning the kinetics of the catalytic reduction of ethylene (22) is considered. Beeck found that the rate of reduction of ethylene bore a definite relation to the lattice distances in the various catalysts which he employed. Applying this information to the reductions of the two compounds examined in this work, one may picture the overall rate of reduction as being the sum of two reactions, one of which produces a threonine derivative, the other, an allothreonine derivative. The magnitude of these competitive rates will then be proportional to the amounts of threonine and allothreonine formed. Since the rate of reduction is a function lattice distance, at least in the case of ethylene, then the relative amount of threonine, which is proportional to its relative rate of formation, might also be expected to be a function of the lattice distances.

For the relative amount of threonine to be ~~proper-~~ *equal* ~~tional~~ to the relative rate of its formation, the reactions producing threonine and allothreonine must be of the same order. Although this was believed to be very likely, reductions of the acetamido compound with platinum oxide and ruthenium on carbon catalysts were taken to half

TABLE VI

RELATIVE AMOUNTS OF THREONINE PRODUCED BY
 REDUCTION OF ETHYL α -ACETAMIDOACETOACETATE AND ETHYL
 O-METHYL- α -OXIMINOACETOACETATE OVER METAL CATALYSTS
 AND SHORTEST DISTANCES BETWEEN CATALYST ATOMS

Metal	% Threonine from Acetamido Compound	% Threonine from O-Methyl Oximino Compound	Shortest Distances Between Atoms
Pt	12 PtO ₂ 20 Pt(O)	37 PtO ₂	2.769
Ir	33	35	2.709
Os	33	34	2.700(2.670, 2.730)
Rh	46	41	2.684
Ru	32	36	2.672(2.645, 2.699)
Cu	14	--	2.551
Co	62	43	2.503(2.499, 2.507)
Ni	24	36	2.487

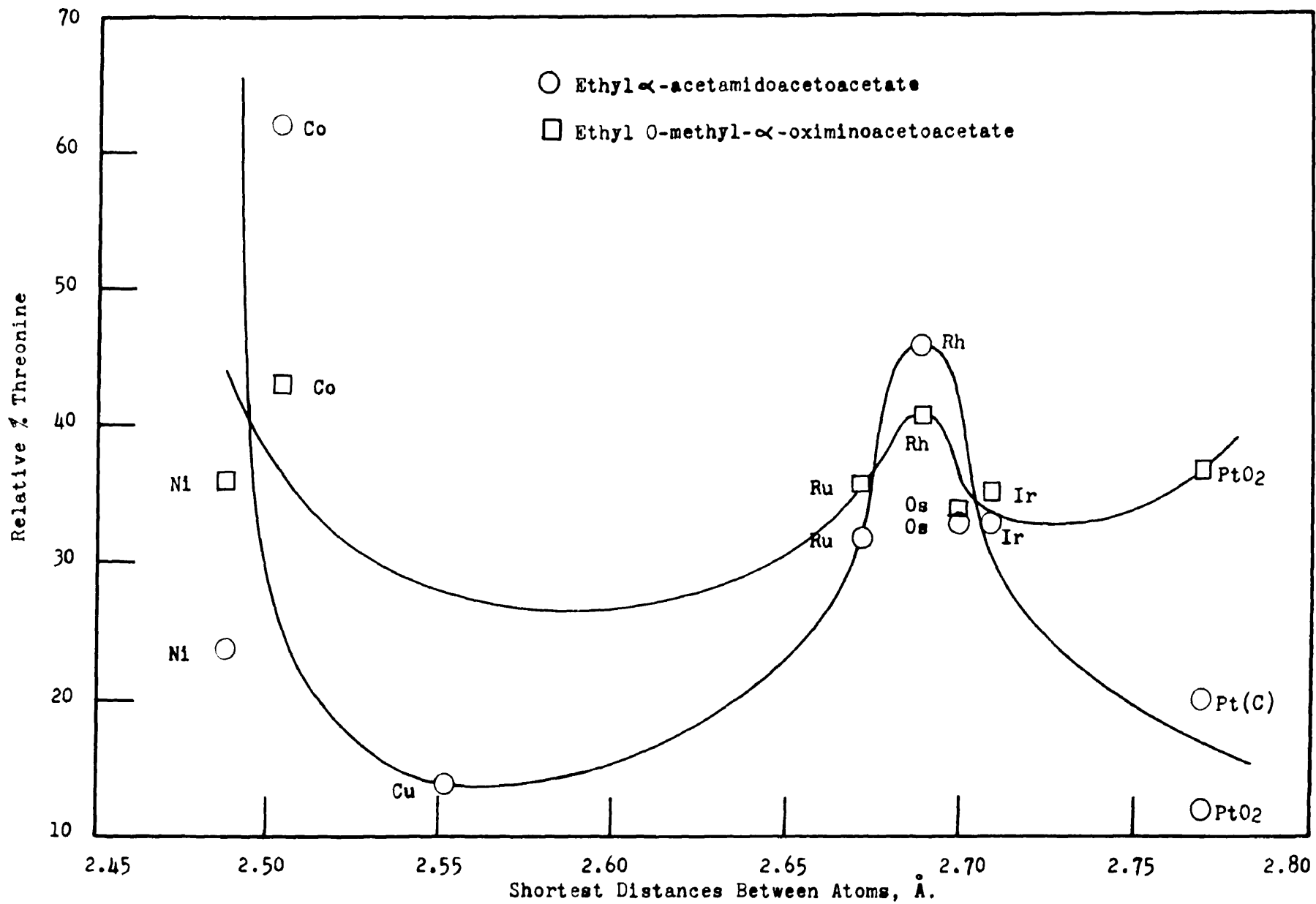


Figure 1. Variation of relative % threonine with lattice parameters of catalysts.

completion and the product analyzed to prove this point. In the case of platinum oxide, 12% of threonine was formed in both the reaction taken to completion and that taken only half way. The unfinished ruthenium reduction yielded a product containing 30% threonine, while that taken to completion gave a sample of mixed amino acids analyzing for 32 % threonine. These results show the independence of the amount of threonine formed with regard to the extent of reaction, and prove that the order of the reaction forming threonine is the same as that forming allothreonine. A replicate ruthenium reduction taken to completion contained 33% threonine.

Since the reductions were carried out under widely varying conditions, the effect of solvent, temperature and pressure on the relative amount of threonine formed also had to be considered. Evidence that changing the solvent has no effect was given by the platinum oxide reductions of the acetamide compound in water and ethanol. In both cases the samples analyzed for 86% allothreonine as based on the total weight of the sample.

Pressure was also without effect on the amount of threonine formed, all other factors being held constant. Platinum oxide in water at one and forty atmospheres gave 12% and 11% threonine respectively. Iridium on carbon at one and forty atmospheres yielded samples both containing 33% threonine.

The effect of changing the temperature which might

be expected to cause large variations in composition was also found to be negligible. Ruthenium on carbon at 25° and at a mean temperature of 65° gave 32% and 35% threonine respectively. Although only this one check was run Attenburrow, Elliot and Penny (14) stated that the amount of threonine produced by reducing ethyl α -benzamidoacetate with Raney nickel was unaltered by changing the temperature from 80° to 160°, which serves as additional evidence.

These various check runs are included in Table I so that they may be compared more conveniently with the other reductions of the acetamido compound.

No runs were made with ethyl O-methyl- α -oximinoacetate to prove the independence of the amount of threonine produced with regard to solvent, pressure, temperature and degree of completeness of reduction.

The variations in conditions for this compound were not as great as those for the acetamido compound, since ethanol was always used as a solvent and pressure was varied only about three-fold. The very close similarity between the curves in Figure 1 for the two compounds is in itself strong evidence that the same phenomena are involved in their determination.

It will be noted that platinum oxide and platinum on carbon catalysts with the acetamido compound yielded different amounts of threonine. A possible explanation of this apparent discrepancy lies in plotting the amount

of threonine versus the shortest distances between atoms. Although these distances have been accurately determined, they bear only a rough relation to the number and magnitude of the parameters which actually effect reduction. These distances are probably not the only distances capable of catalyzing reduction for a given metal. The average distances between adjacent and near adjacent atoms varies in different crystal faces. Thus for a face centered cubic system the average distance between atoms is less in the (111) face than it is in the (110) face. The (111) face has been shown to be predominate in platinum on carbon catalysts (23). If a more even distribution of faces occurs in platinum made by the reduction of platinum oxide, then the average of the exposed distances in platinum on carbon will be shorter. Since platinum falls in a region where there is an increase in threonine formation for a decrease in the distance between atoms, the shift is in the correct direction with regard to the explanation given. The problem is in reality even more complicated because the rate of reduction varies on different faces (22) and probably varies from one distance to another in even the same face.

The anomalous behavior of cobalt and nickel may be explained in a similar way. Since the shortest distances between atoms are nearly the same for these metals, the average of the distances involved in reduction might

easily have a reverse order. A second contributing factor possibly may be the different crystalline systems to which nickel and cobalt belong.

As has been stated, this reduction study was undertaken with the object of finding a new synthesis for threonine. There is a possibility that the mixture prepared by reducing ethyl α -acetamidoacetoacetate with Raney cobalt, which contains 62% threonine, may be separated by recrystallization of the sodium salts of the amino acids as described by Pfister, et al. (13). The mixture itself may be useful in the preparation of synthetic diets for use in nutritional work. It should be possible to prepare this mixture in large quantities with an estimated overall yield of 40-45% based on acetoacetic ester.

EXPERIMENTAL

Catalysts. The platinum oxide catalyst was purchased from the American Platinum Works, Newark, New Jersey.

The platinum, palladium and rhodium on carbon catalysts were prepared by the reduction of the corresponding metal chlorides in acid solution in the presence of active carbon (Darco G-60). One gram of carbon was used for each 0.1 g. of metal chloride dissolved in 25 ml. of approximately 0.2N hydrochloric acid. The reductions were carried out at room temperature with

hydrogen at one atmosphere pressure.

The ruthenium on carbon catalyst of low activity was prepared by reducing ruthenium chloride in the presence of active carbon with hydrazine hydrate by the method of Pollitt (24).

The ruthenium on carbon catalyst of higher activity and the iridium on carbon catalyst were prepared by identical procedures. Three-tenths grams of the metal chloride was dissolved in 25 ml. of water and 3 g. of active carbon was added to the solution. A solution of 2 g. of potassium acetate and 5 g. of potassium carbonate in 25 ml. of water was added to the first solution. The reduction of the chlorides was carried out in a glass liner at 100° with a hydrogen pressure of 65 atmospheres. An hour was allowed for the reduction, but a shorter period of time may be sufficient. The catalysts were filtered from the solution and washed with distilled water.

Osmium on carbon was prepared by shaking a solution of 0.2 g. of osmium tetroxide in 40 ml. of water in the presence of 2 g. of active carbon for one hour at 100° and a hydrogen pressure of 65 atmospheres.

The Raney nickel was made by the procedure of Pavlic and Adkins (25). The other Raney catalysts were prepared from the corresponding aluminum alloys by the procedure used for the preparation of Ni-7 Raney nickel (26). The alloys were ground in an iron mortar to a

powder which would pass a 100-mesh sieve. The cobalt-aluminum (40:60) alloy was purchased from the Gilman Paint and Varnish Company. Devarda's alloy (50% Cu, 45% Al, 5% Zn) was used in the preparation of Raney copper (27). The iron and vanadium alloys were prepared by adding the corresponding metals in granular form to molten aluminum at 1000°. Both of these alloys contained approximately 25% of the catalyst metal.

Ethyl α -Acetamidoacetoacetate. This material was prepared by the reductive acetylation of ethyl α -oximinoacetoacetate (9) with zinc dust and acetic acid in the presence of acetic anhydride, according to the directions of Albertson, et al. (19); b.p. 116-117° (1-2mm.). After recrystallization from an equal volume of acetone by cooling in a Dry Ice-acetone bath the material melted at 48-49°.

Ethyl α -Propionamidoacetoacetate. To a cooled solution of 88 g. (0.55 mole) of ethyl α -oximinoacetoacetate in 400 ml. of propionic acid and 176 ml. of propionic anhydride, 160 g. of zinc dust was added with stirring at such a rate that the temperature was maintained at 40°. When the addition of the zinc dust was about half complete, the reaction mixture became very thick, and water and additional propionic anhydride were added so that stirring could be continued. After all of the zinc dust had been added, the temperature of the reaction was maintained at 40° for twenty minutes by varying

the level of the surrounding ice-bath. Then 450 ml. of water was added and stirring was continued for two hours at room temperature. The mixture was filtered and the excess zinc was washed with 200 ml. of water. The combined filtrate and washings were extracted with four 175 ml. portions of chloroform and the organic layer was washed with two 150 ml. portions of water. The chloroform was removed by distillation under reduced pressure, and the residual oil was distilled in a von Braun flask. After a small forerun, the bulk of the material distilled at 106.5-107° (0.5 mm.). The yield was 93 g. (84% of the theoretical amount). After recrystallization from two volumes of acetone with cooling in a Dry Ice-acetone bath, the material had a melting point of 57-58.5°. Albertson, et al., (19) reported a melting point of 57° for this compound prepared in a similar way.

Ethyl α -Succinamidoacetoacetate. To a cooled, well-stirred solution of 79.5 g. (0.50 moles) of ethyl α -oximinoacetoacetate and 125 g. (1.25 moles) of succinic anhydride in 500 ml. of glacial acetic acid, 200 g. of zinc dust was added portionwise. The temperature was kept at 40-45° by the addition of the metal. After all of the zinc had been added, the reaction mixture was held at 40° for an hour by heating in a water-bath. Then 500 ml. of water was added and stirring was continued for two hours at room temperature. The reaction mixture was filtered and the excess zinc dust was washed

well with water. The filtrate and washings were extracted with six 150 ml. portions of chloroform, and the combined extracts were washed with two 100 ml. portions of water. Chloroform was distilled from the extract under reduced pressure. Solid appeared in the residue when its volume was about 75 ml. and distillation was stopped. After cooling in an ice-bath, the mixture was filtered, yielding 18.5 g. of material.

The aqueous layer was then saturated with sodium chloride and extracted with three 200 ml. portions of chloroform. On reducing the volume of this extract to about 100 ml. and cooling, 28 g. of additional material were obtained. The combined yield from the two extracts was 38% of the theoretical amount. After recrystallization from acetone the material had a melting point of 110-113°. A sample recrystallized twice from methanol and once from acetone melted at 113-114.5°.

Anal. Calcd. for $C_{10}H_{15}O_6N$: C, 48.98, H, 6.17; N, 5.71. Found: C, 49.22, 48.85; H, 6.17, 6.17; N, 5.88, 5.90.

On treating an aqueous solution of the compound with a saturated solution of 2,4-dinitrophenylhydrazine in 2N hydrochloric acid, a yellow 2,4-dinitrophenylhydrazone was obtained. After recrystallization from ethyl acetate the derivative melted at 195-196°.

Anal. Calcd. for $C_{16}H_{19}O_9N_5$: C, 45.18; H, 4.50; N, 16.47. Found: C, 45.11, 45.25; H, 4.65, 4.48; N,

16.41.

Ethyl O-Methyl- α -oximinoacetoacetate. This material was prepared by a procedure identical to that of Adkins and Reeve (9) for ethyl O-ethyl- α -oximinoacetoacetate with the exception that the α -oximinoacetoacetic ester, made by nitrosation of ethyl acetoacetate, was etherified with dimethyl sulfate. At ordinary temperatures, the compound is a colorless oily liquid; b. p. 121-122° (29 mm.); m. p. 22.5°; d_4^{25} 1.085; n_D^{25} 1.4422.

Anal. Calcd. for $C_7H_{11}O_4N$: C, 48.55; H, 6.40; MRD 42.40. Found: C, 48.35, 48.50; H, 6.41, 6.38; MRD 42.25.

General Procedure for the Reductions. All of the reductions run at atmospheric pressure were carried out in a 250 ml. hydrogenation flask fitted with a standard taper joint. The hydrogen absorbed was measured by a gas burette. The reductions at pressures greater than one atmosphere were run in a steel hydrogenation vessel having a void of 300 ml., the pressure drop as read on a gauge being used as a measure of the hydrogen absorbed.

General Procedure for the Isolation of Threonine-Allothreonine Mixtures from the Reduction Products of Ethyl α -Acetamidoacetoacetate and Ethyl α -Propionamidoacetoacetate. After completion of a reduction, the catalyst was removed by filtration and, if the solvent was water, a volume of concentrated hydrochloric acid equal to that of the filtrate was added. The acid

solution was then refluxed for four hours. If the solvent used in the reduction was ethanol, an equal volume of 6N hydrochloric acid was added and the solution was distilled slowly until the temperature rose to 100°. The residual acid solution was refluxed for three hours.

After the acid hydrolysis, the reaction mixture was taken to near dryness under reduced pressure while heating to no more than 50°. The gummy residue of hydrochlorides was taken up in a small quantity of water and lithium carbonate or hydroxide was added until the pH was approximately 6. The solution was filtered and ten volumes of absolute ethanol were added. The solution was allowed to stand until no more solid precipitated, as long as a week being required in some cases. The crude amino acids were filtered and washed with a small amount of 95% ethanol and finally dried in a vacuum desiccator over calcium chloride.

This procedure was altered slightly for the zinc chromite and Raney iron and vanadium reduction products which contained a volatile base. After evaporation of the hydrolysis mixture, an excess of lithium hydroxide solution was added so that the volatile base could be removed by distillation. The excess lithium hydroxide was then neutralized with hydrochloric acid and alcohol added as before. Glycine was the only product isolated from these latter reductions and after one recrystallization from water and alcohol it was identified by mixed

solubility temperatures with an authentic sample of glycine having a solubility temperature of 41.5° (amino acid:water, 1:3). Zinc chromite product, sol. temp. 40.0° , mixed sol. temp. 40.8° ; Raney iron product, sol. temp. 40.4° , mixed sol. temp. 41.2° ; Raney vanadium product, sol. temp. 40.3° , mixed sol. temp. 41.3° .

General Procedure for the Isolation of Threonine-Allothreonine Mixtures from the Reduction Products of Ethyl α -Succinamidoacetoacetate. After removal of the catalyst by filtration, a volume of concentrated hydrochloric acid equal to that of the filtrate was added to the lithium salt solution. After refluxing for four to five hours, the volume of the solution was reduced by distillation under reduced pressure. Toward the end of this distillation, succinic acid began to separate and the distillation was stopped. The solution was cooled in an ice-bath and the succinic acid was filtered and sucked as dry as possible. The volume of the filtrate was further reduced until only a gummy residue remained. The residue was taken up in 10-15 ml. of water and taken to an approximate pH of 6 with solid lithium hydroxide. After filtration to remove any foreign solid, ten volumes of absolute ethanol were added and the solution was allowed to stand until the precipitation of solid was complete.

The initial precipitates were found to contain lithium succinate which was removed by dissolving the

samples in 10 ml. of water and adding an excess of a saturated solution of lead acetate. The insoluble lead succinate was filtered, and excess lead ion was removed by precipitation with hydrogen sulfide. After filtering to remove the lead sulfide, ten volumes of absolute ethanol were added to reprecipitate the amino acids as before.

General Procedure for the Isolation of Threonine-Allothreonine Mixtures from the Reduction Products of Ethyl O-Methyl- α -oximinoacetate. The alcoholic solution from the reduction was filtered to remove the catalyst and the filtrate was added to 300 ml. of water for each 0.10 mole of the initial compound. The mixture was boiled for four hours in an open beaker on a hot plate and water was added occasionally so that the volume did not fall below 100-200 ml. After being filtered, the hydrolysis mixture was concentrated under reduced pressure. Any solids or gums that appeared during the first three-quarters of this operation were filtered. After concentration to near dryness, ten volumes of absolute ethanol were added. After precipitation of the amino acids was complete, they were filtered, washed with 95% ethanol and dried in a vacuum desiccator.

Analysis of Threonine-Allothreonine Mixtures. All mixtures were analyzed for threonine and allothreonine by a solubility procedure which will be described in the next section of this thesis. Neutral equivalents were determined by means of formal titration on all

samples to serve as a check on the identity of the products.

THE ANALYSIS OF THREONINE-ALLOTHREONINE MIXTURES

INTRODUCTION

The development of an analytical method for threonine-allothreonine mixtures was a necessary prerequisite to the study of new syntheses of threonine or to the evaluation of separation methods for threonine-allothreonine mixtures. At the time this work was initiated two microbiological assays for L-threonine had been reported in the literature (28, 29). The disadvantage of these methods for the analysis of the threonine mixtures is that they determine only L-threonine with an accuracy of about 5-10%. Thus the amount of threonine would be obtained by doubling the amount of L-threonine found, and allothreonine would have to be estimated by difference. In addition a microbiologist is needed to properly control such a method. It was therefore hoped that a straightforward physical or chemical method of analysis could be developed.

A large number of procedures have been described for the analysis of amino acid mixtures with the end view of most investigators being the analysis of protein hydrolysates (30). Many of these methods make use of the principles of chromatography and partition chromatography and several of the more promising of these were tried. None of the methods tried were satisfactory and

an analytical procedure based on solubilities was finally developed to solve the problem. By this latter method, both threonine and allothreonine can be determined rapidly with a reasonable degree of accuracy.

CHROMATOGRAPHY AND PARTITION CHROMATOGRAPHY OF THREONINE-ALLOTHREONINE MIXTURES

Discussion and Results. Considerable success has been achieved in the separation of the neutral amino acids by methods of partition chromatography employing liquid-liquid distribution columns. The paper strip technique of Consden, Gordon and Martin (31) is based on partition chromatography, and was tried using n-butyl-alcohol-water and phenol-water systems. In their procedure a strip of humidified filter paper serves as a stationary column of water. A milligram or less of the mixture of amino acids is placed at one end of the paper and the second liquid moves by capillary attraction over the amino acid sample and along the strip of paper. Most amino acids migrate at different rates. The bands of separated amino acids are detected by developing the paper with ninhydrin. Several attempts utilizing this type of analysis were made to separate threonine-allothreonine mixtures, but no noticeable separation was effected.

The method of Wieland and Fremery (32) based on the partition chromatography of the copper complexes of amino

acids on a silica gel column with a phenol-water system also failed to separate the threonine mixture. A modification of this procedure utilizing granular starch as the column substrate and a mixture of methyl cellosolve, n-butyl alcohol and water as a solvent for the complexes was likewise unsuccessful.

The starch column method of Stein and Moore (33, 34, 38) was thoroughly investigated, but it also failed to effect a separation which was clean-cut enough to be useful for analytical purposes. This procedure involves the introduction of a few milligrams of amino acid into a granular starch column, followed by development and elution with wet n-butyl alcohol. Separation is effected by the partition of the amino acids between the wet butyl alcohol and the water phase held stationary by the starch. Samples of the eluate were analyzed for amino acid content and elution curves were prepared from these data. The analyses were performed by heating the unknowns and a standard sample of known concentration with a large excess of ninhydrin, and the intensities of the colors so produced were compared.

Three columns were prepared and 5 mg. of pure threonine, 5 mg. allothreonine and 10 mg. of 50:50 mixture were introduced into them. The elution curves for the two identical columns charged with the pure amino acids is shown in Figure 2 and that for the mixture in Figure 3. Figure 2 indicates that the maximum concentration

of threonine was reached at 1160 ml., while that of the allothreonine was reached at 1120 ml. Figure 3 may be interpreted as showing two peaks, one due to threonine, and one due to allothreonine. It is believed that the analyses were sufficiently precise so that these data are reliable and that a slight separation of the two components was effected. Unfortunately, whatever separation was obtained was too small to be useful for analytical purposes.

It will be noted that the area under the allothreonine curve in Figure 2 corresponds to less than the 5 mg. introduced into the column, while the area under the threonine curve corresponds to more than the 5 mg. in the sample used. This is believed to be due to the use of a mixture of threonine and allothreonine as a standard. This standard was chosen before it was realized that threonine produces the characteristic ninhydrin color more rapidly than does allothreonine under the same conditions. The position of the peaks in the curves should however be unaltered by the failure to use an exact standard.

Sanger (35) has shown that the N-2,4-dinitrophenyl (DNP) derivatives of the natural amino acids may be prepared quantitatively without the occurrence of any racemization. He also demonstrated that these derivatives may be separated, with the exception of leucine and isoleucine, by means of partition chromatography on

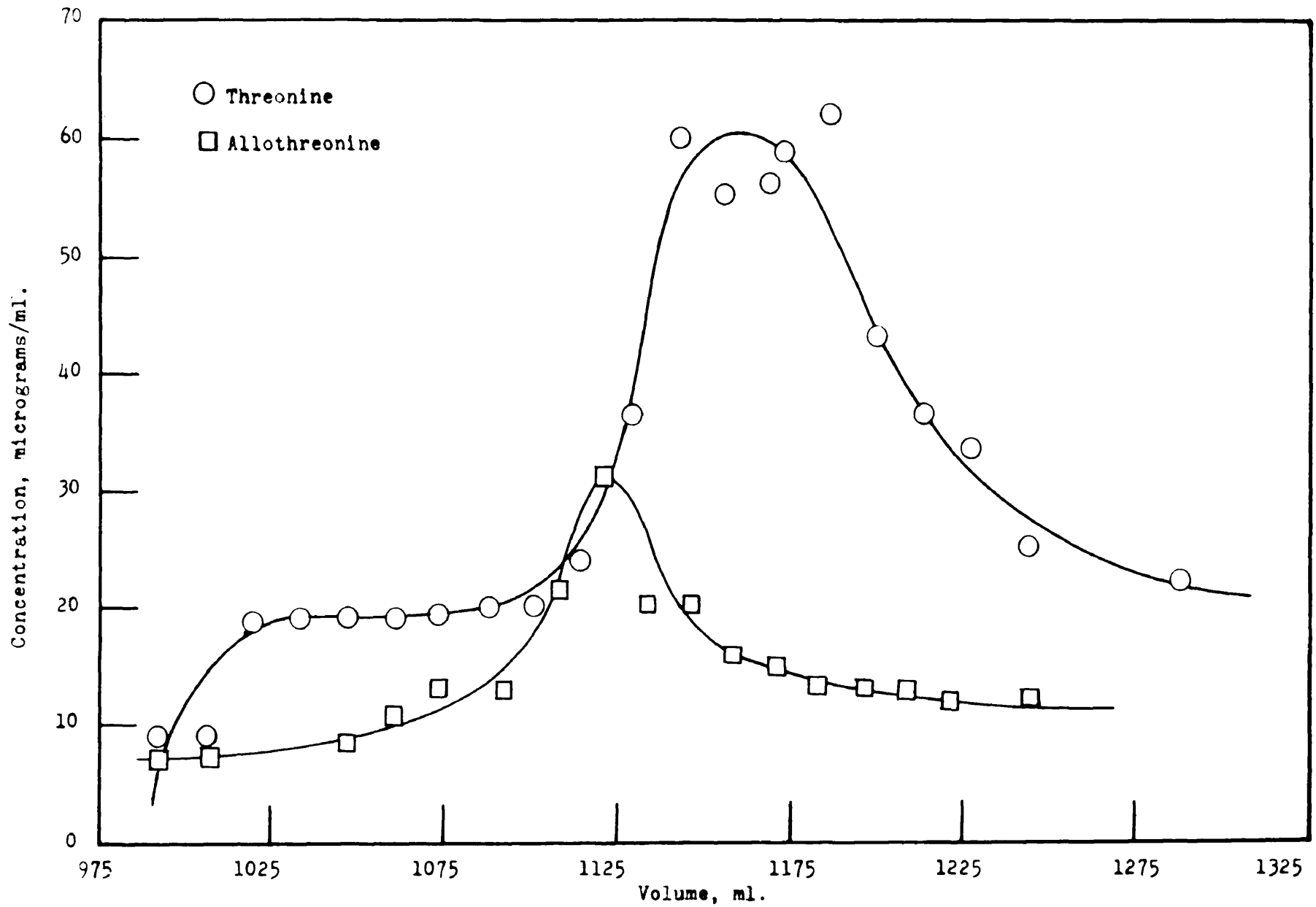


Figure 2. Elution curves for individual samples of threonine and allothreonine.

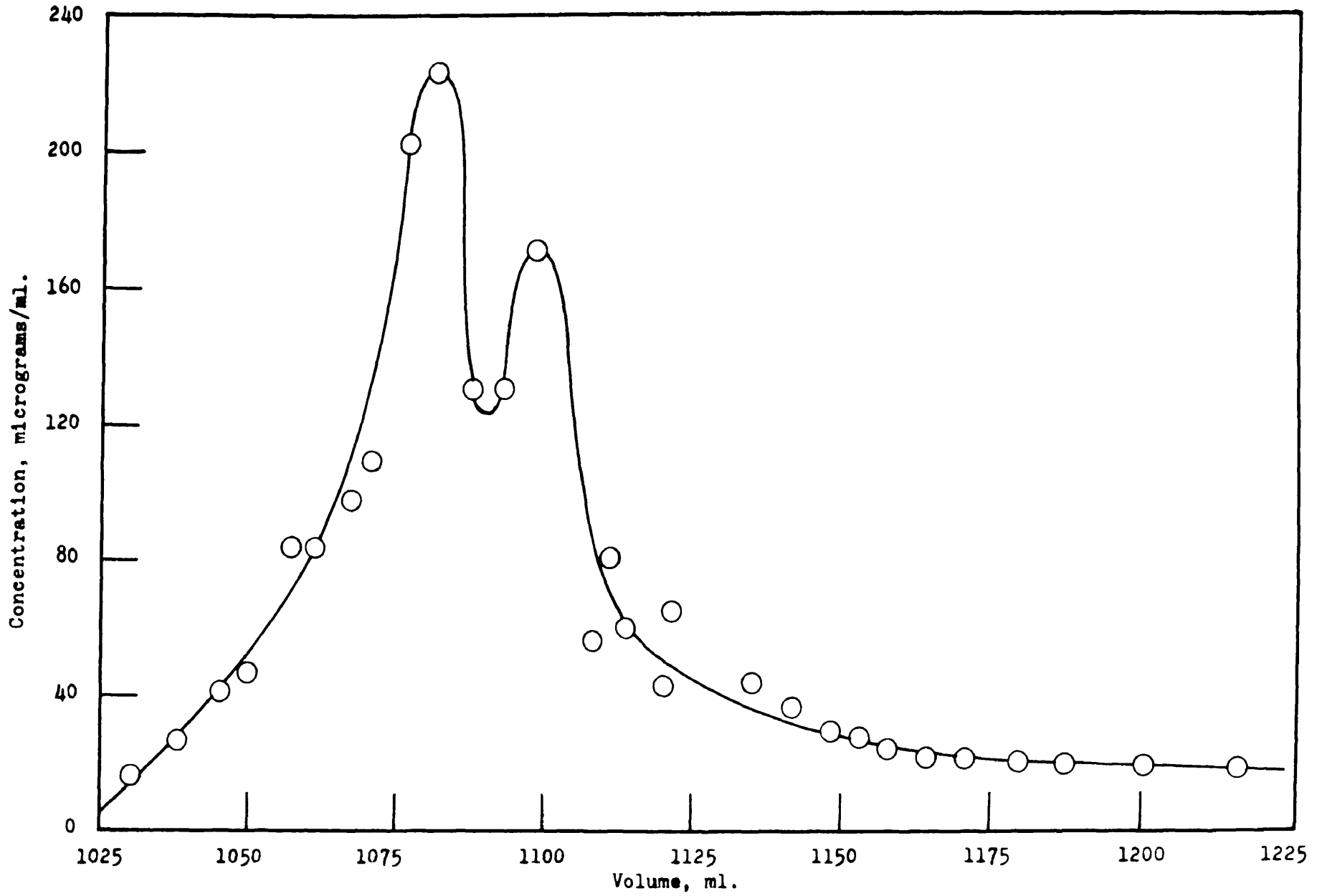


Figure 3. Elution curve for threonine- allothreonine mixture.

silica gel using several solvent combinations.

In view of these facts, the DNP derivatives of threonine and allothreonine were prepared with the idea of separating them by some chromatographic method which might be useful for the analysis of threonine mixtures. Sanger states in his paper that little success was achieved using partition chromatography with starch granules or filter paper to hold the stationary phase, or with adsorption chromatography. Since no indication of the extensiveness of these trials appears in his paper, it was decided that adsorption chromatography and starch partition chromatography should be examined as well as the successful partition chromatography on silica gel columns.

Preliminary testing showed the DNP compounds to be quite insoluble in benzene, carbon tetrachloride, and petroleum ether (boiling range 60-80°); moderately soluble in ether and chloroform; and very soluble in water, methanol, ethanol, and acetone. For the adsorption chromatograms, chloroform was found to be a convenient solvent for placing the derivatives on the adsorbents. A number of adsorbents of widely differing activity were tried and these are listed below in what appears to be the order of decreasing adsorptivity for the DNP derivatives.

Strong:-

Alumina

Alumina treated with dilute

acetic acid

Florosil

Magnesol-Cellite 5:1

Silene EF-Cellite 5:1

Silica gel - Davison

Intermediate:- Drierite

Silicic acid

Cellite

Weak:-

Granular potato starch

Sucrose plus 3% corn starch

The group of adsorbents designated as strong held the derivatives firmly even when the developing solvent was 1:1 ethanol-chloroform by volume. The color of the bands changed with time on the Magnesol and on both samples of alumina, possibly indicating some type of reaction with either the solvent or the adsorbents. Cellite was mixed with the Magnesol and the Silene EF in order to get a satisfactory rate of flow through the columns. Starch and sucrose were too weak and the passage of the derivatives through these adsorbents was unretarded. The intermediate adsorbents held the DNP derivatives from chloroform and the bands formed migrated when 5% of ethanol was added to the solvent. In none of these cases was any separation noted. A mixed solvent of petroleum ether (boiling range 60-80°) and ether (1:1) was substituted for chloroform with alumina, drierite, silicic acid and silica gel, but the results were essentially

unaltered.

The chromatography of either DNP-threonine or DNP-allothreonine on undried Merck silicic acid gave two bands. That this was caused by an uneven distribution of moisture throughout the column was shown by drying the silicic acid by heating in boiling chloroform or by the passage of large amounts of solvent through the column, after which only one band was observed.

The columns for partition chromatography on granular starch were prepared by slurring the starch with the solvent being used. The slurry was poured into the column and solvent was then passed through the column for four days to ensure the establishment of equilibrium before the sample was introduced. The following solvent mixtures were tested:

- I Cyclohexane-n-propanol-water (100:100:13)
- II n-Butanol-water (85:15)
- III Cyclohexane-ethanol-methanol (100:7:13)

The ratios of the compounds in the mixtures were chosen so that the solvents were nearly saturated with respect to water or methanol at room temperature.

The band formed by a mixture of the DNP derivatives moved at a satisfactory rate when solvent I was used, but there was no sign of any separation. With solvent II, the sample moved rapidly through the column, the band broadening with passage, but no development was observed. The starch column did not even retard the

DNP derivatives when solvent III was used.

Another group of partition chromatograms were run using silica gel prepared by the acidification of sodium silicate solution as the column substrate. The solvents used were prepared by shaking together the following ratios of materials and separating the two phases.

- I n-Butanol-chloroform-water (100:3:40)
- II Cyclohexane-n-propanol-water (60:3:20)
- III Petroleum ether (boiling range 60-80°)-ether-water (2:1:1)

In addition, Clark and Lubs buffer solutions of pH 3, 5.8, and 9 were used in place of water in the preparation of solvent pair III. The columns were prepared by wetting the silica gel with the water phase and then the sample dissolved in the organic phase was introduced. Development of the columns was carried out by the continued passage of the organic layer.

Solvent combination I permitted the DNP derivative mixture to pass rapidly through the column without causing any separation. When solvent II was used, the band formed by the sample moved very slowly. There was no apparent separation and the band disappeared before traveling the length of the column. This is believed to have been due to esterification by the solvent, since the eluate contained a yellow compound which was not extracted from cyclohexane by 5% sodium bicarbonate solution. With solvent pair III, the band migrated at a satisfactory

rate, but once again no separation was observed. The replacement of water in solvent III by the buffer solutions did not change the results, except for noticeably decreasing the movement of the band through the column when the buffer of pH 9 was used.

The failure of all the methods based on partition chromatography to achieve a practical separation in the case of threonine and allothreonine is not surprising since these methods with the exception of the starch column method of Stein and Moore fail to separate leucine from isoleucine. It will be recalled that a partial separation of the threonine mixture was accomplished by the starch column, which is evidence of its greater resolving power than the other methods tried. With regard to adsorption chromatography, there are many examples in the literature where a partial separation of diastereoisomers has been achieved (36), but complete separation of diastereoisomers is a difficult accomplishment for ordinary chromatographic methods.

Experimental. Materials. The threonine was purchased from the Eastman Kodak Company, who prepare it by the method of Carter and West (8). It was used without further purification. The allothreonine was obtained in the course of the preparation of threonine by Carter's method. The material was recrystallized three times from water before use, m.p. 240-241°. A threonine-allothreonine mixture, containing 30-40% threonine, was prepared

by the reduction of ethyl O-ethyl- α -oximinocetoacetate according to the directions of Adkins and Reeve (9).

The copper complexes of threonine and allothreonine were prepared by boiling a solution of the amino acids with an excess of copper carbonate. The unused copper carbonate was removed by filtration and the aqueous solution of the complexes was used directly in the experiments.

The DNP derivatives were prepared from threonine and allothreonine by the reaction of the amino acid with 2,4-dinitrofluorobenzene in a sodium bicarbonate solution according to the procedure of Sanger (35).

N-2,4-Dinitrophenyl threonine, recrystallized from water, m.p. 181.5-183°.

Anal. Calcd. for $C_{10}H_{11}O_7N_3$: C, 42.11; H, 3.89; N, 14.73. Found: C, 42.30, 42.18; H, 4.00, 4.00; N, 14.61, 14.75.

N-2,4-Dinitrophenyl allothreonine, recrystallized from dioxane-ether, m.p. 131.5-134°.

Anal. Calcd for $C_{10}H_{11}O_7N_3$: C, 42.11; H, 3.89; N, 14.73. Found: C, 42.21, 42.34; H, 4.01, 4.05; N, 14.93, 15.00.

The sources of the adsorbents used are listed below.

Alumina- "Alorco", 100-200mesh, activated by heating at 200° under reduced pressure for four hours.

Aluminum Ore Company, East St. Louis, Illinois.

Florosil- a magnesium trisilicate, 60-100 mesh,

Floridin Company Inc., Warren, Pennsylvania.

Magnesol- a magnesium trisilicate, Westvaco Chlorine Products Corporation, New York, New York,

Silene EF- a synthetic hydrated calcium acid silicate, Columbia Chemical Division, Pittsburg Plate Glass Company, Barberton, Ohio.

Silica gel- 28-200 mesh, Davison Chemical Company, Baltimore, Maryland.

Drierite- anhydrous calcium sulfate, W. A. Hammond Drierite Company, Xenia, Ohio.

Silicic acid- reagent grade, Merck and Company, Inc., Rahway, New Jersey.

Cellite- "Hyflo", John Mansville Corporation, New York, New York.

Granular potato starch- Amend Drug and Chemical Co., New York, New York.

Sucrose- XXXX powdered confectioner's sugar with 3% corn starch added, American Sugar Refining Company, Baltimore, Maryland.

Silica gel for partition chromatography of the DNP derivatives was prepared according to the procedure of Gordon, Martin, and Syngé (37).

The Paper Strip Technique. A strip of Whatman's No. 1 filter paper (1.5 x 20 cm.), which had been humidified over water in a desiccator for 15 hours, was folded laterally near one end and hung on a short length of glass rod, so that the short end of the suspended strip

dipped in a small glass trough filled with either phenol or n-butyl alcohol, saturated with water. A mixture of the organic solvent and water was poured on cotton in the bottom of the desiccator so that the experiment could be run in an atmosphere saturated with respect to the vapor of both liquids. One drop of a solution containing 20 mg. per ml. of a mixture of threonine and allo-threonine (approx. 1 mg.) was then placed on the long end of the paper strip just below the supporting glass rod. The desiccator was closed over a period of several hours. The liquid in the trough slowly moved over the supporting rod and down the strip, drawn by capillary attraction. When the liquid neared the end of the strip, the paper was removed and the solvent allowed to evaporate. The strip was then sprayed with 1% ninhydrin solution to develop the "band" of amino acids. With both the phenol-water system and the n-butanol-water system, the amino acids migrated, but there was no detectable separation into two spots. Decreasing the size of the sample 5-fold was without effect.

Partition Chromatography of the Copper Complexes of Threonine and Allothreonine. A column 1 cm. in diameter and 25 cm. in height of Davison 28-200 mesh silica gel was covered with a saturated solution of phenol in water. Phenol saturated with water was passed through the column until the eluate was homogeneous and for an additional hour to ensure the removal of excess water phase. Five

milligrams of an equimolar mixture of the copper complexes of threonine and allothreonine dissolved in 5 ml. of wet phenol was introduced into the column. The blue band so formed was developed by passage of more wet phenol. The band broadened slightly as it progressed through the column, but showed no sign of separating into its components.

This procedure was modified using granular potato starch and a homogeneous mixed solvent of 52 parts n-butyl alcohol, 13 parts methyl cellosolve, and 35 parts of water by volume. The starch column, of dimensions similar to those of the silica gel column, was prepared by slurring 20 g. of starch with the mixed solvent and pouring the slurry into a tube constricted at one end and closed with a cotton plug. Solvent was passed through the column for two days to ensure the establishment of equilibrium before the sample, consisting of 5 mg. of the complexes in 2 ml. of solvent, was introduced. The band formed migrated in a manner quite similar to that of the band on the silica gel and there was no indication of fractionation.

Partition Chromatography of Threonine and Allothreonine on Granular Starch. Into each of three columns of 19-22 mm. inside diameter were introduced 30 g. of granular potato starch slurried in 60 ml. of a mixture of 85 parts of n-butyl alcohol and 15 parts water by volume. This solvent mixture was then passed through

the columns for four days to ensure the attainment of equilibrium. The height of the starch in the columns was around 30 cm., and the rate of flow of liquid about 3-5ml./hour. A mixture of 5 mg. of threonine and 5 mg. allothreonine was introduced into one of the columns. Five milligrams of each of these components was introduced separately into the other two columns. Samples of the eluate were collected every hour from the columns using a clock driven turntable. The concentration of amino acid in the samples was determined by heating 2 ml. of the sample with a large excess of solid ninhydrin, and comparing the color so produced with that formed by a standard solution of amino acid under the same conditions. The standard solution was prepared from the mixed amino acids yielded by the reduction of ethyl O-ethyl- α -oximinoacetoacetate. The intensities were compared with an American Instrument Company abridged spectrophotometer, using the 580 mu filter. Elution curves were then prepared by plotting the concentration of amino acid against the total volume of eluate. These elution curves have been given in Figures 2 and 3.

Adsorption Chromatography of DNP-Threonine and DNP-Allothreonine. A quantity of adsorbent sufficient to prepare a column 25 cm. in height by 1.4 cm. diameter was slurried with the solvent to be used, and the slurry poured into a constricted glass tube with a cotton plug at the bottom. If necessary, the adsorbent was packed

down by forcing solvent through the column under the pressure created by a rubber bulb. The sample, consisting of a mixture of equal weights of DNP-threonine and DNP-allothreonine in solution was then placed on the column. The chromatogram was then developed by continuing the passage of the solvent. If there was no migration of the material with the pure solvent, then alcohol was added in increasing amounts until the band began to move, and development was carried out with this mixed solvent.

Starch Partition Chromatography of the DNP Derivatives. Twenty grams of granular potato starch were slurried with the solvent mixture being used, and the slurry was poured into a 1.4 cm. diameter tube, forming a column 23-25 cm. high. The solvent was then passed through the column for four days to ensure equilibrium between the two phases before the sample was introduced. In all cases 2 mg. of an equimolar mixture of the derivatives in solution was used. The columns were developed by passage of the mixed solvent.

Silica Gel Partition Chromatography of the DNP Derivatives. Two different procedures were used for the preparation of the columns. In both, about 40 ml. of silica gel (measured dry without tamping) was used giving a column 20-25 x 1.4 cm.

Procedure A: The silica gel was slurried with the water phase of the solvent pair and the slurry was poured

into the tube. The excess aqueous phase was removed by passing the organic phase of the solvent pair through the column until the effluent liquid was homogeneous. The solution of the mixed sample (2 mg.) was then placed on the column and development was carried out with more of the organic phase.

Procedure B: The aqueous phase of the solvent pair was added dropwise to the silica gel while grinding gently in a mortar. When the silica gel began to form small lumps, the addition of the water phase was stopped, and the moist silica gel was slurried with the organic phase. This slurry was introduced into the column, and after it had been packed down by pressure exerted with a rubber bulb, the sample mixture (2 mg.) was introduced.

Solvent pair III was run on columns prepared by both procedures. Columns for solvents I and II were made using procedure A. The buffered solvent pairs were used on columns prepared by procedure B.

SOLUBILITY ANALYSIS OF THREONINE-ALLOTHREONINE MIXTURES

Introduction. Solubility procedures have been used by many investigators to establish the identity of materials (39) and to analyze mixtures of closely related substances (40). Where conventional melting points fail to prove conclusively the identity of two materials because of decomposition, solubility measurements

may often be substituted. Likewise, where thermal analysis of binary mixtures by means of melting point-composition diagrams is not feasible for similar reasons, the use of a solubility method should frequently prove satisfactory.

Such a solubility procedure was found to be applicable to the analysis of mixtures of threonine and allo-threonine. The analysis was accomplished by first determining the temperature at which the last of a sample dissolved in a fixed amount of water, and then finding the composition which corresponded to that temperature from a previously constructed phase diagram. A ratio of three parts of water to one part of sample was convenient since the temperature extremes were slightly above room temperature but below 100° . It was necessary to use sealed tubes in order to prevent any composition changes during the course of the analysis.

Procedure. Approximately 300-350 mg. of finely ground sample were accurately weighed into a 15 x 75 mm. test tube with care being taken that no solid adhered to the walls of the tube. A weight of water, three times that of the sample, was introduced from a calibrated 2 ml. Koch microburette, making the necessary correction for thermal expansion. The tube was immediately sealed using a small pointed gas-oxygen flame. This could be done satisfactorily if a short length of glass rod was first fused to the lip of the tube. During

this operation the mouth of the tube was pointed away from the flame. To check the accuracy of this procedure for preparing the tubes, several were weighed after sealing, and it was found that the weight of water contained was correct to within 0.1%.

The temperature of complete solution was determined by attaching the tube to a shaft which could be rotated at 60-100 r.p.m. A seven liter battery jar filled with distilled water was used as a bath so that the tube could be immersed three inches below the liquid level and still rotate in a plane only 30° from vertical. This furnished good agitation since the contents of the tube fell from one end to the other as it was rotated. The temperature of the bath was controlled by an internal electrical heating element connected through a Variac. The bath was stirred vigorously to prevent any appreciable temperature differentials. Temperatures were measured by a calibrated 0-100 $^{\circ}$ partial immersion thermometer graduated in tenth-degrees.

As the tube was rotated, the temperature of the bath was rapidly raised to within $2-3^{\circ}$ of the solubility temperature. The rate of heating was then reduced to $0.1^{\circ}\text{C}/\text{min}$. As complete solution was approached, the rotation of the tube was stopped for short intervals so that its contents could be examined more closely. The temperature at which the last crystal dissolved was designated as the "solubility temperature". For tubes prepared

from the same sample this temperature was reproducible to 0.1-0.2°C.

Discussion and Results. The solubility diagram for the analysis was constructed by preparing a series of tubes, containing varying percentages of threonine, from samples of pure threonine and allothreonine. The compositions and solubility temperatures of these tubes are given in Table VII and plotted in Figure 4. Portions of the solubility curves of pure threonine and allothreonine, corresponding to the two legs of the phase diagram, were determined in the same way, and are also plotted in Figure 4. Examination of the figure shows that the solubility of the pure materials is increased by the presence of small amounts of the other component, while larger amounts of the second component cause a decrease in solubility.

It is evident from the "phase" diagram that any temperature below 61.1°, the solubility temperature of pure threonine, may correspond to either of two possible compositions. By the addition of either of the pure components the correct composition may be chosen. Thus the addition of threonine will raise the solubility temperature if the original composition was on the threonine side of the diagram or lower the solubility temperature if the composition was on the allothreonine side. The converse is true if allothreonine is added.

The effect of a third component on the solubility

TABLE VII

SOLUBILITY TEMPERATURES OF THREONINE-ALLOTHREONINE MIXTURES
RATIO OF WATER TO AMINO ACIDS, 3:1

Composition, % Threonine	Solubility Temperature, °C.
0.0	91.2
0.0	91.2
9.9	84.3
20.3	76.8
29.4	69.2
39.1	61.0
50.4	48.6
61.6	33.8
69.6	37.9
76.6	43.3
84.2	49.7
92.1	55.4
100.0	61.2
100.0	61.0

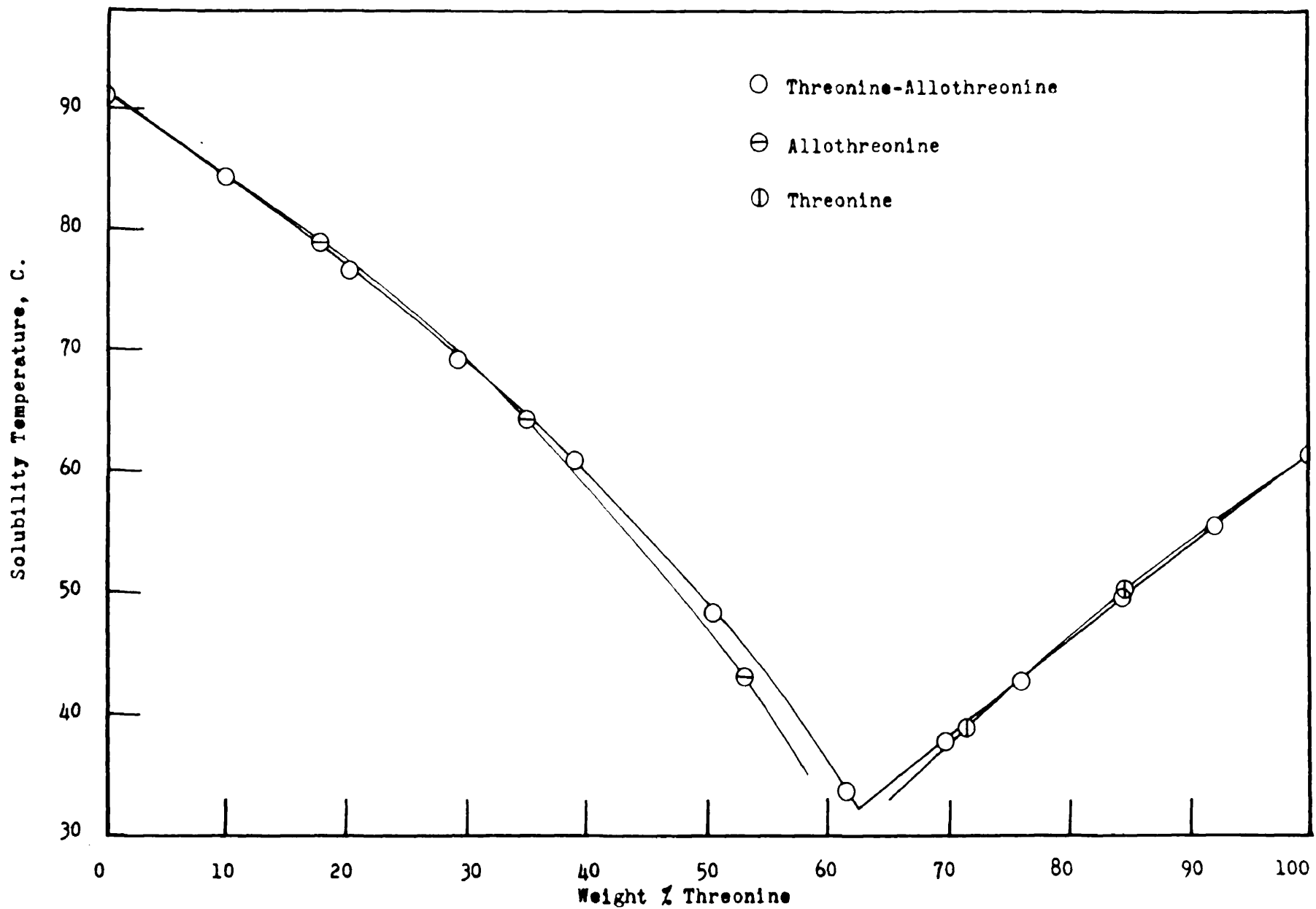


Figure 4. Solubility temperature-composition diagram for threonine-allothreonine system.
 (Ratio of amino acids to water = 1:3)

temperature was studied to see whether analyses of mixtures isolated directly from reactions could be run. In this way any alteration by purification steps of the threonine-allothreonine ratio would be avoided. Glycine and ammonium chloride were chosen to be tested as ionic type impurities because of the possibility of their occurrence in samples. Sucrose was used to determine the effect of neutral impurities on the solubility temperature. The tests were made by preparing tubes containing approximately 80% allothreonine with the remainder of the material being composed of the impurity to be tested. The results of these runs are given in Table VIII and plotted in Figure 5.

TABLE VIII

SOLUBILITY TEMPERATURE OF ALLOTHREONINE IN THE PRESENCE
OF VARIOUS COMPOUNDS

Second Component	Composition, % Allothreonine	Sol. Temp. °C.	Interpolated Sol. Temp. for 80.0% Allo- threonine
None	82.0	78.9	77.4
Threonine	79.7	76.8	77.1
Sucrose	80.7	78.2	77.7
Glycine	80.8	76.3	75.7
Ammonium chloride	79.9	73.1	73.3

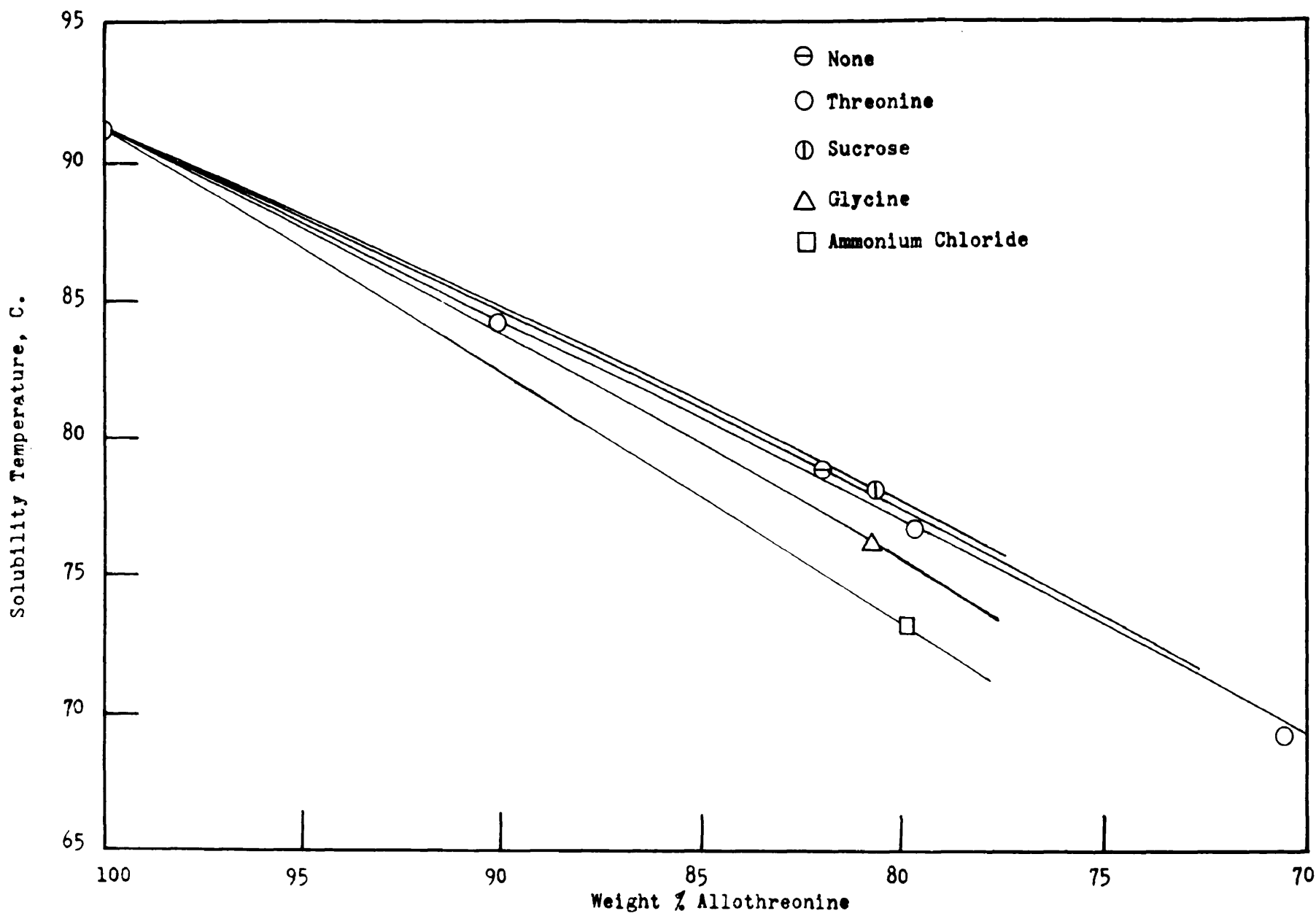


Figure 5. Variation of the solubility temperature of allothreonine in the presence of various compounds.

These data indicate that the presence of appreciable amounts of sucrose and glycine does not cause much error in an analysis. Even ammonium chloride, which caused the most serious deviations, produced only a 5% error when 20% of it was present. In light of this evidence it is felt that results accurate to 2-3 absolute percent were obtained for mixtures composed of 90% or more threonine and allothreonine. However, when materials other than threonine and allothreonine were present, it was necessary to analyze for both amino acids if their ratio was desired. This was done by adding the appropriate pure constituent in such an amount as to change the overall composition enough so that the solubility temperature would fall on the other side of the diagram from which it was on originally. The composition of the original sample was then calculated by the following equation, which is for the case in which pure threonine was added to the sample:

$$P_T = \frac{O_T(W + T) - 100T}{W}$$

where P_T = percent threonine in original sample

O_T = percent threonine observed in mixture of
threonine and sample

W = weight of original sample

T = weight of added threonine.

Closely related to the solubility analysis is the determination of the mutual solubilities of threonine and allothreonine in water at a higher temperature.

This was examined in order to evaluate the feasibility of a temperature-cycle process (41) for the separation of the diastereoisomeric amino acids. The success of such a process is dependent upon a reasonably large variation with temperature of the ratio of the two components in solutions saturated with respect to both. Examination of Figure 4 shows that at 33° a solution saturated with both amino acids has a ratio of 63 parts of threonine to 37 parts of allothreonine. By decreasing the amount of water in relation to the amount of solid the temperature of mutual saturation can be raised. Using a ratio of 5 parts of sample to 6 parts of water, it was found that at 91° the saturated solution contained 62 parts of threonine to 38 of allothreonine. Since the ratio does not change between 33° and 91°, at least within the bounds of experimental error, the complete separation of mixtures of threonine and allothreonine is impossible by controlled recrystallization from water.

The solubility data plotted in Figure 4 for the pure amino acids closely fitted an equation of the form $\log S = a + bt$, where S is the solubility expressed in g./1000 g. of water and t is the temperature in degrees Centigrade. The values of the constants a and b were determined graphically and are given below.

Threonine a = 2.119, b = 0.00660

Allothreonine a = 1.895, b = 0.00683.

The observed solubilities of threonine and allothreonine

and those calculated by the equations are given in Table IX.

TABLE IX

SOLUBILITY OF THREONINE AND ALLOTHREONINE IN WATER

	Temperature, °C.	Solubility, g./1000 g.	
		Obs.	Calcd.
Threonine	39.0	238	238
	50.0	281	281
	61.1	333	333
Allothreonine	43.1	157	155
	64.5	217	217
	78.9	273	274
	91.2	333	333

Materials. The allothreonine was prepared by recrystallization of the mixture of amino acids resulting from the reduction of ethyl O-methyl- α -oximinoacetate and subsequent hydrolysis of the ethyl ester (9). This mixture contains approximately 60% allothreonine. Three-hundred and eighty-six grams of this mixture were dissolved in 770 ml. of water and after two days standing at room temperature the precipitate was filtered and sucked dry. The precipitate weighed 116 g. and was approximately 85% allothreonine. This material was recrystallized once from 300 ml. of water and 100 ml. of absolute ethanol to yield 75 g. of 95% allothreonine.

The crude allothreonine was repeatedly recrystallized by dissolving in the smallest amount of boiling water and adding three volumes of absolute ethanol and allowing the solution to cool to room temperature. After seven recrystallizations, the solubility temperature remained unchanged after two additional recrystallizations and the material was considered pure. The yield after drying in a vacuum desiccator was 41 g. The neutral equivalent was determined by formol titration Calcd. 119.1. Found 119.3.

The threonine was prepared by repeated recrystallization of 20 g. Eastman Kodak Company threonine. The recrystallizations were carried out by dissolving the material in the smallest amount of boiling water and adding three volumes of absolute ethanol and allowing to cool to room temperature. After six recrystallizations the solubility temperature was unchanged by four subsequent recrystallizations. The yield after drying in a vacuum desiccator was 9.2 g. The neutral equivalent was determined by formol titration. Calcd. 119.1 Found 119.4.

SOLUBILITY TEMPERATURES AS A MEANS OF IDENTIFICATION
AND AS A CRITERION OF PURITY OF AMINO ACIDS

The α -amino acids form a class of compounds which do not give true melting points, but instead decompose upon heating. Decomposition points do not serve as a measure of purity, and as an extreme example, it has been found that a mixture containing 30% threonine (m.p. 228-229° dec.) and 70% allothreonine (m.p. 251-252° dec.) gives a decomposition point corresponding to that of pure threonine.

The success of solubility measurements in analyzing threonine-allothreonine mixtures suggested that the same procedure might be usefully employed in determining the purity or the identity of certain other amino acids. By far, the greatest use of such a procedure would be the determination of purity. The solubility method would also be a help in identification. In addition, mixed solubility temperatures corresponding to mixed melting points would make positive identification possible.

The solubilities in water of many of the natural amino acids, including both optically active and racemic forms, have been determined at various temperatures. Examination of these data shows that great variations in solubility exist among these amino acids. The insolubility of certain of them, such as cystine, tyrosine,

thyroxine and dibromotyrosine, precludes the use of this method. On the other hand, proline and hydroxyproline are exceedingly soluble and would require the use of large samples if accurate results were to be obtained. The remaining amino acids have solubilities in a range which should be useful.

It is possible to divide this latter group into two classes based on a suitable ratio of amino acid to water for the determination of solubility temperatures. The two ratios which were chosen were one part amino acid to six and twenty-five parts of water. A sample of 150-200 mg. was used for the 1:6 ratio and 40-50 mg., for the 1:25 ratio. The procedure for preparing the tubes and determining the solubility temperatures was identical to that used in the threonine-allothreonine analysis.

DISCUSSION AND RESULTS

The solubilities of the amino acids to which the solubility temperature method appeared to be applicable have been expressed by equations of the form $\log S = a + bt + ct^2$, where S is solubility in g./1000 g. of water and t is degrees Centigrade. Values of the constants a , b and c are listed in Table X and the mean deviation between observed solubility and that calculated by the equations is included in order that some idea of their accuracy may be obtained. All of these data have been determined by Dalton and Schmidt (42, 43). Included in Table X are

TABLE V

CONSTANTS FOR THE SOLUBILITY EQUATIONS AND CALCULATED
SOLUBILITY TEMPERATURES FOR THE AMINO ACIDS

Amino Acid	a	b x 10 ²	c x 10 ⁵	Mean Dev. %	Calcd. Sol. Temp.
Ratio 1:6					
Glycine	2.1516	1.087	-4.114	0.92	6.7
DL-Alanine	2.0830	0.5608		0.66	24.8
D-Alanine	2.1048	0.4669		0.25	25.1
DL-Serine	1.3432	1.520	-3.548	0.56	68.9
DL-Glutamic acid	0.9317	1.523		3.34	84.7
DL-Valine	1.7749	0.2389	2.607	0.54	92.8
DL-Methionine	1.2597	1.108	-1.221	0.73	97.0
Ratio 1:25					
L-Isoleucine	1.5787	0.07862	2.594	1.26	18.7
L-Phenylalanine	1.2974	0.6982		1.15	43.6
L-Glutamic acid	0.5331	1.613		1.36	66.3
DL-Isoleucine	1.2616	0.2512	3.794	1.33	67.2
DL-Phenylalanine	0.9986	0.5252	3.140	1.04	78.3
DL-Asartic acid	0.4181	2.016	-4.999	0.76	71.4
DL-Norleucine	0.9258	0.4524	3.402	0.66	89.5
L-Tryptophane	0.9156	0.4834	2.988	1.44	90.0
DL-Leucine	0.9013	0.2635	4.591	0.70	98.0

the equilibrium temperatures of complete solution as calculated from these equations.

Deviations of the observed solubility temperatures from the calculated equilibrium temperatures may be expected for two reasons. First, the method is a dynamic one in that the temperature is continually being raised, and this should cause the observed temperatures to be higher than the calculated ones. Second, errors are to be expected in the calculated temperatures, and the magnitude of these can be estimated by use of the mean deviations. Unfortunately, the range of all the equations used was 0-65°, and there is the possibility of much larger errors in the calculated values above 70°.

The actual solubility temperatures of four of these amino acids were determined so that an idea of the agreement with the calculated values might be obtained. Two amino acids from each class were chosen and the results are given in Table XI.

TABLE XI

OBSERVED SOLUBILITY TEMPERATURES OF FOUR AMINO ACIDS

Amino Acid	Solubility Temperature		
	Observed (2 recryst.)	Observed (4 recryst.)	Calcd.
DL-Alanine (1:6)	25.2	----	24.8
DL-Valine (1:6)	94.1	94.4	92.8
DL-Aspartic acid (1:25)	70.7	70.7	71.4
DL-Phenylalanine (1:25)	80.4	----	78.3

The agreement between the calculated and observed temperatures was good for alanine, the small deviation being in the expected direction. The results for valine and phenylalanine were considerably higher than the calculated temperatures. These amino acids are not wetted too well by water, and it is felt that this may possibly have been the cause of the deviation. However, considerable uncertainty exists about the accuracy of the calculated values for these amino acids since the range of the equations has been exceeded. The low values obtained for aspartic acid may be due to an impurity in the sample which was not removed by recrystallization or errors in the solubility data from which the calculated value was obtained.

In addition to these four amino acids, DL-leucine was tried, and although the calculated temperature is 98.0° , the material did not completely dissolve when kept at 100° for thirty minutes. The possible causes of this deviation are the same as those considered for valine and phenylalanine.

Particle size is of great importance in determining the rate of solution so that for those amino acids which do not supersaturate badly it is advisable to dissolve the sample in the tube completely and then chill it with shaking. Small crystals of a more reproducible size are obtained in this way. This procedure was used with valine, phenylalanine and aspartic acid.

MATERIALS

The DL-aspartic acid was purchased from the Eastman Kodak Company. The DL-phenylalanine, DL-valine and DL-leucine were purchased from Mann Fine Chemicals, New York, New York. The DL-alanine was a product of the Bios Laboratories, Inc., New York, New York.

The recrystallizations were carried out according to the procedures given for the individual amino acids by Dunn and Rockland (51).

THE SYNTHESIS OF HYDROXYPROLINE BY THE METHOD OF
FEOFILAKTOV AND ONISCHENKO

DISCUSSION

The synthesis of hydroxyproline was undertaken to supply the Poultry Department of this University with a small amount of this amino acid for nutritional work. Four syntheses of hydroxyproline are to be found in the literature.

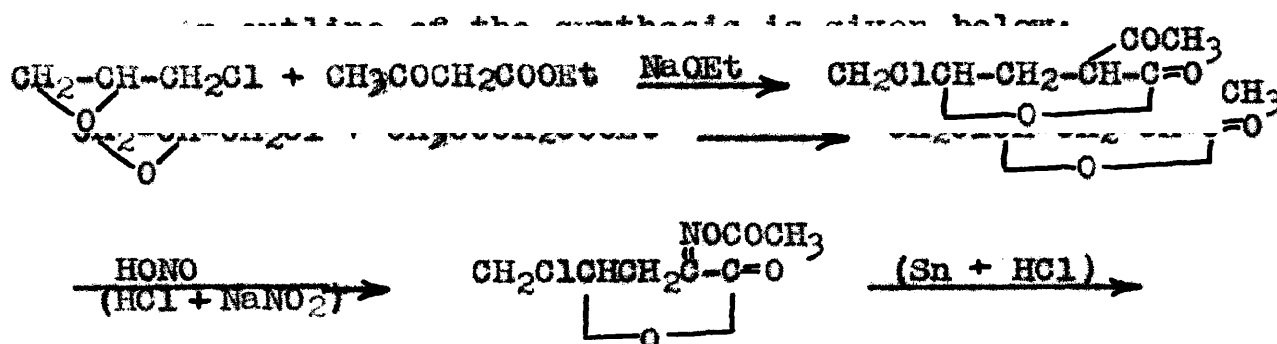
Leuchs and coworkers (44-47) were the first to synthesize this amino acid. Their preparation consisted of reacting epichlorohydrin with sodiomalonic ester to yield α -carbethoxy- δ -chloro- γ -valerolactone, which after chlorination, acid hydrolysis and decarboxylation gave α,δ -dichloro- γ -valerolactone. Treatment of the dichloro compound with aqueous ammonia gave a mixture of the diastereoisomeric hydroxyprolines. This synthesis was modified considerably by Traube, Johow and Tepohl (48), who treated the α -carbethoxy- δ -chloro- γ -valerolactone with ammonia to obtain α -carboxyamido- δ -amino- γ -valerolactone. After hydrolysis of the amide, the lactone was brominated and decarboxylated, and subsequent removal of hydrogen bromide from the resultant α -bromo- δ -amino- γ -valerolactone with moist silver oxide gave the desired product.

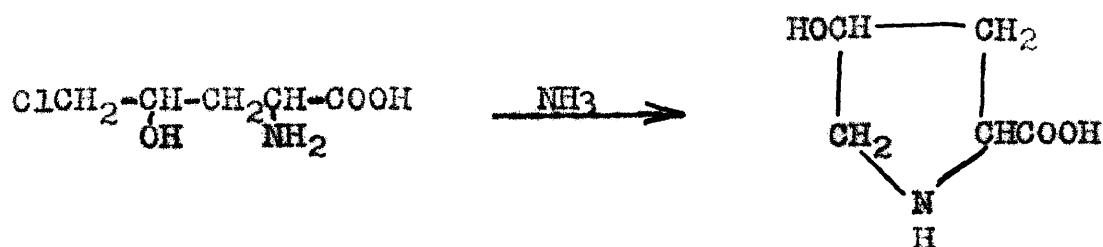
Hammarsten (49) prepared hydroxyproline by treat-

ment of ethyl α -benzamido- γ,δ -dibromovalerate with barium hydroxide, which yielded a mixture of α -benzamido- δ -bromo- γ -valerolactone and O-benzoylhydroxyproline, the latter yielding hydroxyproline on further hydrolysis.

Both McIlwain and Richardson (50) and Feofilaktov and Onischenko (1) prepared hydroxyproline from α -acetyl- δ -chloro- γ -valerolactone, which was in turn made from epichlorhydrin and acetoacetic ester. McIlwain converted the lactone to the α -oximino compound by the use of nitrosyl sulfuric acid, and by reduction with platinum oxide in acetic acid obtained α -amino- γ -hydroxy- δ -chlorovaleric acid, which when treated with ammonia gave a mixture of hydroxyprolines. The Russian workers prepared the α -oximino acetate by nitrosation of the lactone with nitrous acid. Reduction with tin and hydrochloric acid, followed by the action of ammonia yielded the hydroxyprolines.

Despite the lack of experimental details, the method of Feofilaktov and Onischenko was chosen because it appeared direct and better yields were claimed for it than for the other syntheses.





No difficulties were encountered in the acetoacetic ester condensation and the nitrosation reaction. The condensation was carried out essentially by the procedure described by Traube and Lehman (50). The nitrosation was run following the directions of Feofilaktov and Onischenko.

For the reduction of δ -chloro- α -oximino- γ -valerolactone it was hoped that a catalytic method might be used to simplify the isolation of the product. Reduction with platinum oxide in acetic acid was not tried since McIlwain's yield by this procedure from the free oxime was only 30% and the reduction required three days. Hartung's method of reducing oximes with palladium on activated carbon in alcoholic hydrogen chloride failed (17); no hydrogen was adsorbed in three hours time.

Reduction by tin and concentrated hydrochloric acid seemed to be satisfactory since it gave over 50% of the theoretical yield. Actually, the highest yield of the reduction product ever isolated was 27% of the theoretical amount, but if the material was carried on to the next step without isolation, an overall yield of approximately 50% was obtained after the second reaction. It

is believed that partial ring closure to hydroxyproline occurred when the reduction mixture was treated with lead carbonate to remove excess hydrochloric acid.

The ring closure of the δ -chloro- γ -hydroxy- α -aminovaleric acid by ammonia proceeded readily to yield a mixture of the four stereoisomeric forms of hydroxyproline. The use of barium hydroxide, which it was hoped would facilitate the isolation of the hydroxyproline, led to side reactions so that the resulting products consisted mainly of a water soluble oil, which yielded only a few crystals on long standing.

By the reactions described above, it was possible to obtain a mixture of the two hydroxyprolines in approximately a 25% overall yield based on epichlorohydrin, if the intermediate δ -chloro- γ -hydroxy- α -aminovaleric acid was not isolated before cyclization.

EXPERIMENTAL

δ -Chloro- α -acetyl- γ -valerolactone. To a solution of sodium ethoxide formed from 115 g. (5 g. atoms) of sodium metal and 1500 ml. of absolute ethanol was added 1300 g. (10 moles) of redistilled ethyl acetoacetate. After cooling to 0-5°, 463 g. (5 moles) of epichlorohydrin was added rapidly with stirring. The temperature of the mixture rose very slowly. It was allowed to stand overnight and then heated to 45-50° for one hour. The ethanol was removed by distillation at re-

duced pressure, and the residual viscous yellow syrup was neutralized with approximately one liter of 5N hydrochloric acid and a liter of water added. The organic layer was separated from the aqueous layer, and the latter was extracted twice with 200 ml. portions of ether. The extracts and the organic layer were combined, washed well with water, and dried over magnesium sulfate. Ether was distilled from the extract, and the residue was distilled under reduced pressure from a modified Claisen flask. After a forerun of acetoacetic ester, the bulk of material distilled at 135-137° (3mm.). Approximately 50 g. of high boiling material remained in the flask at the end of the distillation. The yield was 554 g. (64% of the theoretical amount) of colorless oil, which slowly turned pink. This material was used without further purification in the preparation of the acetate of δ -chloro- α -oximino- γ -valerolactone.

δ -Chloro- α -oximino- γ -valerolactone Acetate. To a cooled, well stirred mixture of 554 g. (3.14 moles) of δ -chloro- α -acetyl- γ -valerolactone and a solution of 240 g. of sodium nitrite in two liters of water, was added approximately 650 ml. of 5N hydrochloric acid. The rate of addition was controlled so that the temperature did exceed 30°. Before the hydrochloric acid addition was complete, solid began to form in the mixture. The end of the reaction was indicated by the appearance of nitrous fumes. The mixture was cooled to 5° and the

solid filtered and washed with several portions of cold water. The crude oxime acetate was air dried and recrystallized from the smallest possible amount of methanol. The yield of white crystals was 480 g. (75% of the theoretical amount) m. p. 113-114°. Feofilaktov reported a melting point of 115-116° for this compound. A sample of the above material was recrystallized several times from methanol and benzene for analysis.

Anal. Calcd. for $C_7H_8O_4NCl$: C, 40.89; H, 3.92; N, 6.81; Cl, 17.25. Found: C, 41.22, 41.24; H, 4.07, 4.16; N, 6.78, 6.62; Cl, 17.47, 17.52.

δ -Chloro- γ -hydroxy- α -aminovaleric acid. Forty grams of granular tin and 80 ml. of concentrated hydrochloric acid were heated until the evolution of hydrogen became vigorous. The source of heat was removed, and a solution of 50 g. (0.243 mole) of δ -chloro- α -oximino- γ -valerolactone acetate in 150 ml. of 2.5N hydrogen chloride in ethanol was added as rapidly as the condenser could handle the vapors. After the vigorous reaction had subsided, the mixture was kept boiling by heating for ten minutes. After cooling, the excess tin was filtered and the filtrate was concentrated by distillation under reduced pressure until tin salts began to precipitate. The residue was diluted to 700 ml. and lead carbonate was added until the pH was approximately 5. The solution was filtered and hydrogen sulfide was passed in to remove the last traces of lead and tin. The sulfides

were filtered, and the filtrate was concentrated under reduced pressure until solid began to appear. Eight milliliters of aniline was added to the residue, followed by two volumes of ethanol. After cooling in ice, the crystals were filtered and dried in a vacuum desiccator. The yield was 6.6 g. of white crystalline solid (27% of the theoretical amount) melting with decomposition at 173-174° after recrystallization from water and ethanol. Feofilaktov reported a melting point of 165.5-166.5°.

Anal. Calcd. for $C_5H_{10}O_3NCl$: Cl, 21.2. Found: Cl, 20.94, 20.88.

Preparation of the Hydroxyprolines from the Oxime Acetate. Sixty grams (0.292 mole) of δ -chloro- α -oximino- γ -valerolactone acetate was reduced according to the procedure given above. Lead carbonate was added as before until the pH was 5, and the precipitate of lead salts was removed by filtration. One liter of concentrated ammonia was added to the filtrate, the volume of which was nearly two liters. The mixture was allowed to stand for a day, filtered, and boiled until its volume was reduced to 1500 ml. Further concentration to 800 ml. was carried out under reduced pressure. One hundred and five grams of barium hydroxide octahydrate was added and distillation was continued until the distillate was neutral. The residue was diluted to one liter and 33 g. of sulfuric acid, diluted to 150 ml., was added. The barium sulfate was filtered, and the

filtrate was again treated with lead carbonate. Traces of lead were removed with hydrogen sulfide. The light amber solution was concentrated to 50 ml., and 10 ml. of aniline and five volumes of ethanol were added. Crystals formed immediately. They were filtered and dried, yielding 16.5 g. of mixed hydroxyprolines (47% of the theoretical amount), m. p. 236-237° decomp. The mother liquors, after evaporation to near dryness, yielded slightly over one gram of additional material upon the addition of absolute ethanol. Leuchs reported decomposition points of 261° for DL-hydroxyproline and 250° for DL-allo-hydroxyproline (44).

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