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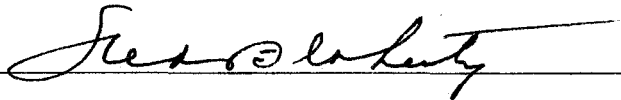
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I hereby recommend that the thesis prepared under my supervision by John H. Howard

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Approved by:



THE ISOLATION
OF HYDROXYPROLINE AND PROLINE
FROM COLLAGEN

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TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
EXPERIMENTAL	2
RAW MATERIAL	2
HYDROLYSIS	3
EXAMINATION OF LITERATURE METHODS	5
ABSOLUTE ETHANOL AS A SPECIFIC SOLVENT FOR PROLINE	7
NITRITION	9
ION EXCHANGE RESIN INVESTIGATION	18
DISCUSSION	24
SUMMARY	26
BIBLIOGRAPHY	27

INTRODUCTION

Proline (1) and hydroxyproline (2) were first isolated from protein hydrolyzates by Fischer early in the current century by esterifying the amino acids present and fractionating these esters by vacuum distillation. Since then, numerous methods have been used to isolate these amino acids. These include n-butanol extraction followed by n-propanol extraction (3), reineckate formation (4), the copper salts' method of Town (5), picrate formation with subsequent decomposition with aniline (6,7), and rhodanilate formation (8). The methods are generally tedious and the amino acid products, both in quantity and quality, leave much to be desired. Recent work has emphasized the use of solvent extraction, especially the patent by Sifferd (9) and the investigation of McCollum and Rider (10). Hamilton and Ortiz (11) have recently prepared the N-nitroso derivatives of proline and hydroxyproline. We were simultaneously and independently working along similar lines. While these investigators reported that their single attempt to isolate proline and hydroxyproline from gelatin with use of the N-nitroso derivatives gave poor yields, we are gratified to state that we were able to obtain good yields of both of these amino acids from collagen in a number of trials.

This research concerns studies for affecting complete hydrolysis of collagen using hydrochloric acid and the determination of the properties of the resulting products when treated with nitrous acid. From this information a unique method for isolating crude proline and hydroxyproline has been developed. This depends upon the fact that all

of the amino acids except proline and hydroxyproline form hydroxy acids when reacted with nitrous acid while these two imino acids stop at the nitrosamine stage. Proline and hydroxyproline are regenerated from their nitrosamines and separated from the hydroxy acids and each other by appropriate solvent separation. Since neither of these amino acids is commercially available at a reasonable price, this research makes a practical as well as a theoretical contribution to our knowledge of the chemistry of collagen.

EXPERIMENTAL

Raw Material

A salt extracted corium split of a steer hide was used as the source of a purified, native collagen for this research. It had been prepared (by P. R. Buechler and L. Anusz in this laboratory) by the following procedure:

A steer hide was obtained from a packing-house immediately after removal from the animal, thoroughly washed, cut into strips, and frozen in a deep-freeze unit. Subsequently each piece was taken out, allowed to thaw a little, and split into layers on a splitting machine. Only the strips from the center of the corium were taken since this portion contains a minimum of elastic and reticular tissues. The center corium strips were cut into very small squares and treated with eight per cent sodium chloride solution for twenty-four hours. This solution was changed once after about twelve hours. In order to prevent bacterial action, one part of merthiolate per 100,000 parts of solution was added to the salt solutions which were kept in a refrigerator at about 10°C during the soaking period. The material

was then rinsed in water and placed in ninety-five per cent ethanol. The preparation was completed by extraction with ethanol and acetone at about 50°C, followed by room temperature drying. The pieces were ground to a coarse powder in a Wiley mill using a 3 mm. screen and stored in a tightly closed container.

Analysis of the powder showed it to contain 15.61 per cent total nitrogen, 0.50 per cent total ash, 0.03 per cent lipids, 0.16 per cent total carbohydrates, 0.10 per cent pentoses, and 12.90 per cent moisture. Neuman (12,13) has given an amino acid analysis of this preparation. His data accounts for all of the total Kjeldahl nitrogen in terms of amino acids' nitrogen and amide nitrogen. Jacobs (14) has reported that this collagen preparation obtains 0.6 per cent of its nitrogen from elastic and blood vessel tissues, and 1.7 per cent of its total nitrogen from globular proteins.

Hydrolysis

Optimum conditions for affecting complete hydrolysis with hydrochloric acid were then studied. The factors considered were time, strength of acid, and ratio of acid to collagen, the total amount of hydrogen chloride being held constant when the first two factors were considered. The results are given in Table I.

Formol titrations and ammonia nitrogen analyses were employed to follow the rate of hydrolysis and for determining when hydrolysis was complete. Optimum conditions were considered to be those conditions under which the collagen was completely hydrolyzed under reflux conditions in the shortest time with the least amount of acid

Hydrolysis of Collagen

With Hydrochloric Acid under Reflux Conditions,

As a Function of Time, Concentration of Acid,

And Ratio of Acid to Collagen

Normality of Acid	Time, hours	Ratio, Acid (ml.) to Collagen (g.)	Formol Nitrogen, per cent*	Ammonia Nitrogen, per cent*
6.0630	1	6:1	11.96	0.55
6.0630	2	6:1	12.93	0.55
6.0630	4	6:1	13.48	0.55
6.0630	5	6:1	13.67	0.56
6.0630	6	6:1	13.67	0.55
6.0630	7	6:1	13.69	0.55
6.0630	8	6:1	13.67	0.55
6.0630	16	6:1	13.69	0.60
6.0630	24	6:1	13.67	0.77
6.0630	1	15:1	12.32	0.56
6.0630	3	15:1	13.52	0.56
6.0630	4	15:1	13.67	0.55
6.0630	5	15:1	13.67	0.55
3.0980	5	12:1	12.62	0.55
3.0980	16	12:1	13.42	0.55
3.0980	24	12:1	13.55	0.55
4.1767	5	9:1	13.24	0.55
4.1767	16	9:1	13.65	0.55
4.1767	24	9:1	13.67	0.55
5.1903	5	7.2:1	13.45	0.55
5.1903	16	7.2:1	13.67	0.55
5.1903	24	7.2:1	13.65	0.56

*Not corrected for moisture. The formol figure 13.67 and the ammonia nitrogen figure 0.55 respectively convert to 15.68 and 0.63 on a dry basis.

to give a maximum constant formol titration value with no increase in the ammonia nitrogen constant. These optimum conditions were found to be refluxing 5 hours (16 hours caused no ill effects) with a ratio of 6 ml. of 6 N hydrochloric acid for each gram of collagen. The ammonia nitrogen constant (amide nitrogen) was found to be 0.63 ± 0.02 per cent and the formol amino nitrogen constant 15.68 ± 0.02 per cent (both values on a moisture-free basis).

Examination of Literature Methods

A survey of the available methods for the isolation of proline and hydroxyproline was made. Fischer's ester method (1,2), Bergman's rhodanilic acid method (8), and the reinecke acid method of Kapfhammer and Eck (4) were deemed too tedious. Town's copper salts' method (5), adopted from an earlier observation of Fischer (1,2), was finally selected as the most practical method. This, in combination with Cox and King's method (6) for decomposing the picrates of mono-amino acids, was finally the way this phase was carried out. The only other variation was the use of hydrochloric acid as the hydrolyzing agent with subsequent concentrating in vacuo followed by diluting and re-concentrating.

According to Town (5), the copper salts of the amino acids fall into three classes. The first class is insoluble in water. The second class is soluble in water but insoluble in absolute methanol. The third class is soluble in both water and absolute methanol, and both proline and hydroxyproline are in this group. Town applied these principles when he isolated proline from wheat gliadin. He hydro-

lyzed with sulfuric acid, removed same with barium hydroxide, prepared the copper salts of the amino acids by adding copper carbonate and heating, finally obtaining an absolute methanol soluble crop of crystals. These were converted to the free amino acids using hydrogen sulfide and an absolute ethanol soluble fraction obtained from these. This was treated with picric acid, extracted with ether to remove the excess picric acid, the ether phase discarded, the picrate residue decomposed with sulfuric acid, the free picric acid removed with ether, the hydrogen sulphate removed, and proline finally obtained as the free compound.

Klabunde (7), in isolating hydroxyproline, proceeded in the same manner as Town (5) up to the point of obtaining the absolute ethanol soluble fraction. She then worked on the fraction which was insoluble in absolute ethanol by converting same to the picrate, washing with ether, decomposing the picrate with aniline and discarding the aniline layer, removing the residual aniline with ether, finally precipitating the hydroxyproline with absolute ethanol.

We encountered considerable difficulty with the hydroxyproline fraction after converting it to the picrate. As a result of this, no attempt was made to work up the crude proline fraction. Ether, even with use of a liquid-liquid extractor for seven continuous days, was found to be ineffective in removing the yellow color (due to the picric acid) from the hydroxyproline fraction after treating with picric acid and aniline. Benzene, Norit-A, collagen, lead oxide, and lead acetate (all used in side experiments) were found to be ineffective in remov-

ing picric acid completely. It was finally found necessary to take to dryness in vacuo and extract with absolute ethanol in the presence of a small amount of hydrochloric acid to finally obtain a white precipitate of presumably hydroxyproline hydrochloride. Five grams of collagen yielded 0.8 grams of crude proline and 0.3 grams of crude hydroxyproline hydrochloride. The proline fraction contained 10.92 per cent total nitrogen, 8.71 per cent total ash, 7.4 per cent moisture, and 15.38 per cent chlorides. The hydroxyproline fraction contained 0.29 per cent total nitrogen, 96.5 per cent total ash, 0.0 per cent moisture, and 58.8 per cent chlorides. No attempt was made to analyze either fraction specifically for proline or hydroxyproline due to their obviously low purity.

The failure to quantitatively remove all of the residual hydrogen chloride previous to copper salts' formation undoubtedly influenced the results. The high ash in the proline fraction was due to an accumulation of soluble inorganic compounds from the reagents used in the process. The method, as carried out, is therefore very poor for isolating these amino acids from collagen.

Absolute Ethanol as a Specific Solvent for Proline

Proline is the only amino acid purportedly soluble in absolute ethanol. Accordingly, a mixture of commercial amino acids, in the proportions found in this collagen by Neuman (12,13), was extracted with absolute ethanol at 22°C to see if that reagent was a specific solvent for proline. Sixteen hundred and fifty milligrams of 1(-) proline were added initially and twenty-six hundred milligrams were recovered from

the mixture! This represents a 170 per cent recovery. If absolute ethanol is a specific solvent for proline, either the amino acids contained proline as an impurity or the ethanol was not an absolute grade (most of the amino acids are soluble to some degree in ethanol-water systems).

The purity of the absolute ethanol was investigated first. The dielectric constant of absolute ethanol at 20°C is given as 25.8 (15). Using air, benzene, chloroform, pyridine, and acetone in a high frequency oscillator to establish the curve, the dielectric constant of the absolute ethanol used in the above work was found to be 26.0. Absolute ethanol from a freshly opened container gave a reading of 25.2; U. S. P. 95 per cent ethanol gave a reading of 28.5. This definitely showed that the absolute ethanol in either case contained less than 5 per cent water.

Refractionation of the recovered proline by dissolving in absolute ethanol and adding drop-wise to ether yielded an ether insoluble fraction and an oily, brown, acetone-ether-ethanol soluble fraction, the last being insoluble in hot and cold water, and therefore obviously not an amino acid(s). The refractionated proline contained 12.52 per cent total nitrogen which is a little higher than 12.17 per cent, the theoretical value, but no worse than the 11.79 per cent found for the original proline. The slightly high value is to be expected since most of the amino acids are probably slightly soluble in absolute ethanol at this temperature, and most of the amino acids present contain more nitrogen than proline. The hydroxyproline used in the orig-

inal mixture contained 13.41 per cent total nitrogen (against 10.68 per cent, the theoretical value) and 7.07 per cent of the same was found to be soluble in absolute ethanol. This could quite logically be proline since one is usually a contaminant of the other in commercial preparations. Furthermore proline has a unique nitrogen content. It is more than coincidence that the nitrogen content of the refractionated proline approached that of pure proline. Although the material was not analyzed specifically for proline, it would appear to be practically pure proline with a small amount of impurities due to other amino acids which have a higher nitrogen content. On these bases, it would appear that absolute ethanol is a specific solvent for proline and the abnormal proline recovery was due to that amino acid being an impurity in the other amino acids used in the mixture.

Nitritation

Proline and hydroxyproline are secondary amines. The other amino acids are primary amines. According to accepted methods of organic chemistry, secondary amines may be separated from primary amines as follows:

a-Treating with nitrous acid

b-Ether extraction to remove both the nitrosamines formed by the reaction of nitrous acid with the secondary amines and the deaminates or hydroxy compounds formed by the reaction of nitrous acid with primary amines.

c-Subsequent hydrolysis of this mixture with dilute hydrochloric acid to decompose the nitrosamines and con-

vert them to the amine hydrochlorides without attacking the hydroxy compounds

d-Ether extraction to remove the hydroxy compounds leaving behind the amine hydrochlorides

e-Subsequent recovery of the amine hydrochlorides by removal of the solvent

These principles were applied to both commercial proline and hydroxyproline. Both yielded ill-smelling, yellow oils after treatment with nitrous acid, ether extraction, and removal of the ether from the ether phase. These oils, after hydrolysis at 121°C (15 lbs. steam pressure) with 6 N hydrochloric acid, yielded colorless, transparent films on removal of the solvent.

In the preliminary work, 0.3000 gram of l(-) proline was dissolved in 4 ml. of concentrated hydrochloric acid, placed in an ice-bath, cooled to 0°C, 0.5 gram of sodium nitrite added slowly over a five minute period, held at 0°C for five minutes, and allowed to warm up to 22°C over a period of ten minutes. Water (5 ml.) was added to the mixture and the same was held at 22°C for fifteen minutes. The solution was then extracted twice with 20 ml. aliquots of ether for ten minute intervals. After removal of the ether from the combined ether phases, a small amount of yellow oil remained. This was ampuled with 4 ml. of 6 N hydrochloric acid and held at 121°C for nine hours. The ampule was then opened, contents removed, and taken to dryness on a steam-bath. After drying in vacuo for sixteen hours at 60°C, a colorless, transparent film resulted. It weighed 0.0064 gram and contained

8.96 per cent total nitrogen. This compares favorably to the theoretical total nitrogen in proline hydrochloride which is 9.25 per cent. The yield converts to 1.62 per cent of the theoretical value.

A similar investigation was carried out with l(-) hydroxyproline. In this case, 0.1000 gram of the material was dissolved in 2 ml. of concentrated hydrochloric acid, placed in an ice-bath, cooled to 0°C, 0.2 gram of sodium nitrite added slowly over a five minute period, held at 0°C for thirty minutes, gradually allowed to warm up to 23°C over a ten minute period, and held at 23°C for thirty minutes. The solution was then extracted twice with 10 ml. aliquots of ether, and the ether removed from the combined ether phases to yield a small amount of yellow oil. This was then treated in the same manner as that from the proline. The recovered, dry material weighed 0.0024 gram which converts to a 1.88 per cent yield. It was found to contain only 4.83 per cent total nitrogen (hydroxyproline hydrochloride contains 8.37 per cent total nitrogen). This was not too surprising since the original hydroxyproline contained 13.41 per cent total nitrogen (the theoretical value is 10.68 per cent) indicating it to be highly impure. Further investigation of conditions for increasing nitrosamines' production was confined to proline alone for that reason. It was felt that conditions increasing proline nitrosamine production would equally apply to hydroxyproline since the substances are so similar.

In still another trial, 0.2000 gram of l(-) proline was dissolved in 10 ml. of water, 0.4 gram of sodium nitrite added and dissolved, cooled to 0°C in an ice-bath, 1 ml. of concentrated hydrochloric

acid added, mixed, held at 0°C for thirty minutes, allowed to warm up to 23°C over a ten minute period, and held at 23°C for one hour. After ether extraction and ether removal, the nitrosamine was hydrolyzed as before. This time the yield was 3.83 per cent of the theoretical and the recovered material contained 8.98 per cent total nitrogen. The progressive increase in yield as a function of increase in temperature of nitriting indicated that a study of higher temperatures during nitriting should be made.

When nitriting was carried out at 23°C (all the other conditions being the same as in the above procedure except that the amount of sodium nitrite was doubled), the recovered material contained 8.97 per cent total nitrogen and the yield was 18.84 per cent of the theoretical. When nitriting was carried out at 60°C for 1.5 hours and the solution extracted with ether after hydrolyzing at 121°C with 6 N hydrochloric acid, the recovered material contained 9.24 per cent total nitrogen and the yield was 73.85 per cent of the theoretical. Removal of the ether from this last ether extract showed a fair quantity of nitrosamine present. Thus all of the nitrosamine had not been converted to the hydrochloride. Had all of the nitrosamine been converted to the hydrochloride, recovery would probably have been close to 100 per cent. In summary, it was found that maximum nitrosamine production could be obtained by using 1 N hydrochloric acid for nitrous acid generation and nitriting at 60°C for 1.5 hours.

The next step was to apply the above information to a decolorized, collagen hydrolyzate. A rancid, yellow oil was obtained after

nitritation and subsequent ether extraction. Refluxing the yellow oil with 6 N hydrochloric acid for nine hours caused little change in color so a little spongy palladium was added and refluxing continued for sixteen hours. At the end of this time the material was a mahogany color and had an ethereal odor so it was discarded since this was obviously not the desired course of reaction (the mixture should have become colorless and odorless). It thus appeared that the nitrosamines were stable when boiled with 6 N hydrochloric acid.

Having shown that nitrosamines could be obtained from a collagen hydrolyzate, the next step was to try to improve nitrosamines' hydrolysis conditions. A collagen hydrolyzate was nitrited, nitrosamines extracted with ether, and stored in 6 N hydrochloric acid at 4°C. Aliquots of this solution were then used in this phase of the work. Time, temperature, and amount of 6 N hydrochloric acid were the factors studied. None of the products resembled a synthetic mixture of proline hydrochloride and hydroxyproline hydrochloride prepared from commercial amino acids and which were demonstrated to be stable compounds. Hydrolysis at 121°C for 16 hours and using a ratio of 100 ml. of 6 N hydrochloric acid to 1 ml. of nitrosamines' concentrate seemed to be the optimum conditions. However, the product had a very high ash content (about 30 per cent). In attempting to explain this ash content, the reagents were found to contribute about 60 per cent of the ash found. Boiling after nitriting (as a means of removing all of the remaining nitrous acid) caused elimination of practically all nitrogenous material. Addition of urea (to decompose residual nitrous

acid) and subsequent boiling after nitriting lowered the ash content but the chloride and nitrogen contents could not be rationalized with the solids' content. These experiments suggested that the previous conclusion that the nitrosamines of proline and hydroxyproline could not be decomposed by boiling with 6 N hydrochloric acid was in error. Three new experiments were carried out. The essential process, except for minor variations, was as follows:

Collagen (5 grams) is hydrolyzed by refluxing for 16 hours with 30 ml. of 6 N hydrochloric acid, decolorized by boiling with one gram of Norit-A for fifteen minutes, the carbon removed and discarded, and the filtrate further acidified by adding 15 ml. of concentrated hydrochloric acid. The volume is then adjusted to 270 ml. with water. The solution is then heated to 60°C and 90 ml. of a solution of sodium nitrite (pre-heated to 60°C and containing 7.2 grams of sodium nitrite) is slowly added. After thorough mixing, the solution is held at 60°C for 1.5 hours with shaking every fifteen minutes. The solution is then simmered for one hour, most of the solvent removed by straight distillation, concentrated to a slush on a steam-bath, extracted four times with 12.5 ml. aliquots of concentrated hydrochloric acid to leach out the proline hydrochloride and hydroxyproline hydrochloride, and concentrated to dryness in vacuo. The residue is dissolved in water, residual hydrogen chloride removed with silver carbonate, residual silver ion removed with hydrogen sulfide, concentrated in vacuo, dried in vacuo at 60°C, and extracted with absolute ethanol (100 ml. for each gram of proline expected). The insoluble hydroxy-

proline is filtered off and dried in vacuo at 60°C for 16 hours. The ethanol extract is taken to dryness in vacuo under 60°C as a result of which the white crystals of proline remain on the bottom of the dish and the gunk collects on the sides.

By the above procedure a maximum yield of 0.335 gram of crude hydroxyproline was obtained. Neuman and Logan (13) report a value of 13.3 per cent hydroxyproline for this collagen. On the basis of this figure the above maximum yield of hydroxyproline is 57.8 per cent of theory.

A maximum yield of 0.1058 gram of crude proline was likewise obtained. Neuman (12) reports a value of 16.5 per cent L(-) proline for this collagen. The above maximum yield thus converts to 14.7 per cent of the theoretical value.

The analyses of the crude fractions of proline and hydroxyproline obtained from the three experiments are given in Table II. Hydroxyproline was determined by a modification of the method of Neuman and Logan (13). This method is based on the intensity of the lavender color developed when hydroxyproline is oxidized with hydrogen peroxide in the presence of cupric ions in an alkaline medium and coupled with p-dimethylaminobenzaldehyde at 70°C in an acid medium. A Beckman Spectrophotometer was employed to read per cent transmission after color development and a wave-length of 560 millimicrons was selected since maximum absorption was found to take place there rather than at 540 millimicrons as recommended by the original investigators. L(-) proline was determined microbiologically by the method of Dunn, et al.

TABLE II

Analyses* of Crude Fractions of Proline and Hydroxyproline

Isolated from Collagen with Use of Nitrous Acid,

And Employing Silver Carbonate to Remove Residual Hydrogen Chloride

Fraction	Ash	Chlorides	Ammonia Nitrogen	Total Nitrogen Found	Total Nitrogen Calculated**	1(-) Proline	Hydroxyproline
Hydroxyproline (Expt. #1)	16.1	0.0	0.00	5.63	5.55	8.6	42.1
Proline*** (Expt. #1)	7.0	0.3	0.00	9.11	7.84	50.9	15.4
Hydroxyproline (Expt. #2)	13.6	0.0	0.00	6.65	7.54	16.6	51.7
Hydroxyproline (Expt. #3)	11.9	0.0	0.00	7.75	7.60	17.3	51.5
Proline*** (Expt. #3)	7.1	1.4	0.00	9.40	7.00	47.7	11.2

*in grams per hundred grams of material dried 16 hours at 60°C in vacuo

**calculated from the per cent nitrogen in the per cents proline and hydroxyproline as found by analysis

***analysis for hydroxyproline was made on a different part of the sample which accounts for the discrepancy between the nitrogen found and calculated figures

(16). This method is based upon the amount of lactic acid produced by the test organism, *Leuconostoc mesenteroides* P-60, when grown for 72 hours at 35°C in a completely synthetic medium, aliquots of which contain known amounts of proline which the organism requires for growth. Total ash, chlorides, total nitrogen, and ammonia nitrogen were determined by the usual micro-techniques. All of the samples were dried in vacuo for 16 hours at 60°C, weighed, dissolved in water, diluted to a specific volume, and aliquots used for the various analyses.

It is obvious that none of the fractions are pure. The presence of proline in the hydroxyproline in each case is not too surprising since we made no attempt at further purification of the same. There may have been insufficient ethanol present to remove all of the proline or several extractions should have been made with fresh ethanol each time. Inability to make a material balance (ash + 1(-) proline + hydroxyproline + ammonia = 100) indicates that organic impurities are still present. These are probably the deaminates which, acting as weak acids, could quite conceivably account for the presence of hydroxyproline in the proline. The appreciable quantities of ash present pose a problem. Qualitative tests indicated the ash in every case to be essentially a phosphate. This probably came from the vegetable charcoal, used in the various decolorizing treatments. This carbon contained 2.24 per cent ash which was mostly phosphates. The practically complete absence of chlorides seems to indicate that silver carbonate treatment is an effective means of removing chloride ions.

The excellent material balance in regard to total nitrogen in

the case of the hydroxyproline fractions seems to indicate that the methods of analysis are reliable. It would also appear that racemization of neither amino acid had taken place during processing. This observation appears to be a positive one in the case of the proline, and by analogy hydroxyproline, though no positive proof has been established in the case of the latter. A nitrogen balance was not expected for the proline fractions since practically all of the best material was used for the other analyses and only the scrapings of the dishes were available for the hydroxyproline analyses.

Ion Exchange Resin Investigation

Synthetic resins are reputed to be effective agents for anion and cation removal. The main draw-back to their use is the fact that the concentration of the ion(s) to be removed must be very low (about 0.01 N) which means large volumes of liquid must be employed, considerable time is consumed during de-ionization, and the de-ionized effluent, if it contains the desired material(s), must be reconcentrated, usually in vacuo, which again is not a fast operation. Even with these complicating factors, it was deemed advisable to attempt a large scale experiment with use of an anion exchange resin to remove residual hydrogen chloride in order to improve the yields of proline and hydroxyproline both in quantity and quality, the theory being that these amino acids, being such weak acids, would not be retained on the column, but would pass on through, and only the hydrogen chloride and possibly the deaminates of the rest of the amino acids remain behind.

Fifty grams of collagen were hydrolyzed in the manner previously

described. After decolorization, an attempt was made to remove the glutamic acid present. The material was concentrated in vacuo to 100 ml. and saturated with hydrogen chloride at 4°C for one hour. Only a slight crystal crop formed. Fifty ml. of concentrated hydrochloric acid were added and hydrogen chloride passed for another 30 minutes, this time surrounding the container with a salt-ice bath. No increase in the crystal crop took place although 1800 grams of hydrogen chloride were used in toto. Storage at 4°C for 24 hours did not increase precipitation. The crystal crop was removed, saturation with hydrogen chloride repeated (450 grams were used over a 40 minute period) using a salt-ice bath, and the material stored for 90 hours at 4°C. Another crystal crop had formed, about equal in volume to the first. The preparation was held an additional 66 hours at 4°C in an attempt to increase the size of this crop. This was unsuccessful. The crystals were removed, dried, and combined with the first crop. Analysis showed them to be sodium chloride and not l(+) glutamic acid hydrochloride since they were composed of 61.2 per cent chlorides, 0.00 per cent total nitrogen, and 100.0 per cent ash. This would seem to indicate that the separation of l(+) glutamic acid, as its hydrochloride, from a collagen hydrolyzate requires a quite critical concentration of solids during saturation with hydrogen chloride. No further work was done on that problem due to it being only a minor feature of the process.

After removing the supposedly l(+) glutamic acid hydrochloride crystals, the liquid was allowed to warm up to room temperature, nitrated, concentrated to a slush, the slush extracted with concentrated

hydrochloric acid, and this extract taken to dryness in vacuo, all in the manner previously described. This residue was completely soluble in concentrated hydrochloric acid as well as absolute ethanol (300 ml.). It was taken up in water, decolorized, diluted to 10 liters with water, and passed through an Amberlite IR 4 column which was 79.5 cm. in height and 2.2 cm. in diameter in the manner prescribed by the manufacturer of the resin (17). The column was regenerated with 4 per cent ammonium hydroxide after 3000 ml. of the preparation had percolated through. The pH of the last aliquot of this cut was 4.0. Another 2500 ml. of material was passed through the column before it was regenerated. The pH of the last aliquot of this fraction was 5.5. The rest of the solution was then passed through the column, regenerating after each 2500 ml. portion had percolated through, the pH of the last aliquot of each fraction being 5.5 and 5.6 respectively. The iso-electric point of proline (18) is at pH 6.3 and that of hydroxyproline (18) is at pH 5.82. A solution having a pH of less than 5.82 would therefore contain acidic materials other than these two amino acids. Therefore the column was regenerated from time to time in order to minimize the amount of these contaminants. All of the effluents were combined and concentrated to about 150 ml. by vacuum distillation. This concentrate was an orange-brown color. It should here be mentioned that none of the cuts were without a trace of color as they emerged from the column, although the affluent was water-white. The above mentioned concentrate was decolorized to a pale straw color with two treatments of Norit-A. Vacuum distillation and drying yielded a mahogany colored, viscous

mass. This was taken up in 200 ml. of water, decolorized, and added drop-wise to 900 ml. of acetone. A white precipitate formed but this coalesced to an orange colored mass which was amorphous, even after holding at 4°C for 40 hours. The acetone layer was discarded (it was a clear, yellow liquid). The residue began to show crystal growth on removal of the acetone. Drying in vacuo at 60°C for one hour yielded a reddish-brown gunk, devoid of crystals. Extraction with 200 ml. of absolute ethanol yielded a cream colored powder. This was re-extracted with absolute ethanol, dissolved in water, decolorized, and taken to dryness in vacuo. The weight of this fraction was 1.1416 grams. The analysis is given in Table III coded Hydroxyproline Crop #1. This material was white and slightly hygroscopic.

The combined ethanol extracts from the preceding were taken to dryness in vacuo, taken up in water, decolorized, and taken to dryness in vacuo. The product was a soft, yellow, transparent mass. It was extracted with absolute ethanol and yielded a residue which was a light-brown colored powder. This was taken up in water, decolorized, concentrated to about 2 ml., and a white precipitate obtained by adding 75 ml. of absolute ethanol and 50 ml. of ether. This material was cream colored and more hygroscopic than the first crop of hydroxyproline. It weighed 0.2813 gram. Its analysis is given in Table III coded Hydroxyproline, Crop #2.

The absolute ethanol extract, after the removal of the second crystal crop of hydroxyproline, was a pale straw color. It was concentrated to about 2 ml. in vacuo. This was a pale yellow colored

TABLE III

Analyses* of Various Fractions of Hydroxyproline and Proline

Isolated from Collagen with Use of Nitrous Acid,

And Employing Amberlite In 4 to Remove Residual Hydrogen Chloride

Fraction	Ash	Chlorides	Ammonia	Total Nitrogen,		1(-) Proline	Hydroxyproline
				Nitrogen Found	Nitrogen Calculated**		
Hydroxyproline (Crop #1)	12.7	0.0	0.00	6.35	5.09	9.7	36.6
Hydroxyproline (Crop #2)	18.3	0.0	0.00	6.02	5.33	7.9	40.9
Proline (Crop #1)	11.5	0.0	0.00	8.78	8.81	19.0	60.8
Proline (Crop #2)	28.1	0.0	0.00	5.41	2.97	15.1	10.6

*in grams per hundred grams of material dried 16 hours at 60°C in vacuo

**calculated from the per cent nitrogen in the per cents proline and hydroxyproline as found by analysis.

syrup. It was taken up in 25 ml. of warm absolute ethanol and added drop-wise to 25 ml. of ether. A white precipitate formed but this rapidly turned to an orange color. The preparation was held 20 hours at 4°C. The clear, orange colored supernatant was discarded, the residue dissolved in 200 ml. of absolute ethanol, and added drop-wise to 200 ml. of ether. A white precipitate formed but this again rapidly turned to an orange color. Recrystallization in a similar manner yielded a viscous, orange colored mass when all of the ether and ethanol were removed from the same. (The treatment of the supernatant is given in the next paragraph). Extraction of this residue with absolute ethanol yielded 0.0900 gram of a light pink colored powder which was slightly hygroscopic. Its analysis is given in Table III coded Proline, Crop #1. The ethanol extract from this phase yielded a reddish-brown film on drying so it was discarded.

The ether-ethanol solution remaining from the above treatment was concentrated to a brown tar. This was dissolved in 25 ml. of absolute ethanol and added to 50 ml. of acetone. A white curd separated. This was filtered off and it rapidly darkened to a brown color even over calcium chloride in a desiccator. The dry product weighed 0.0641 gram. Its analysis is given in Table III coded Proline, Crop #2. The acetone-ethanol supernatant was discarded.

The preceding was given in such detail in order to show the many obstacles that were met, seemingly at every step of the process. No such difficulties were encountered when silver carbonate was used to remove residual hydrogen chloride. It is obvious that none of the

fractions are pure. Organic material is still present as evidenced by the inability to make a material balance. The ash content of each fraction is still quite significant. Qualitative tests again indicated the ash in all cases to be essentially a phosphate. The Norit-A was probably the main contributor of this component since it was found to contain 2.24 per cent ash on an as is basis. This ash also gave positive response to the various phosphate tests.

A nitrogen balance can only be obtained in the case of the first crystal crop of proline and it is chiefly hydroxyproline! This would seem to indicate that either racemization of the proline had occurred (the microbiological assay reports only the natural isomer) or the Amberlite IR 4 contributed nitrogen to the products and this last is quite logical since it is an aniline-formaldehyde condensation product. It would further appear that the Amberlite IR 4 either removed most of the proline or the nitrogen-containing compound contributed by the same catalyzed the degradation of the proline, as a result of which so little of that amino acid was recovered.

The combined yield of hydroxyproline, even including the first crop of proline, is less than that obtained in any case when silver carbonate was used to remove residual hydrogen chloride. Further, the purity is considerably lower.

DISCUSSION

It cannot be questioned but that this research has yielded an entirely new and novel method for isolating proline and hydroxyproline from collagen, and quite conceivable other proteins. The method

is not perfect in any sense but the yields, when silver carbonate is used to remove residual hydrogen chloride, are good and a many-fold concentration of each of the desired amino acids has been obtained. The writer concentrated on trying to increase the yields and did not have the time available to thoroughly investigate means of purifying the crude products. Suffice it to say, considerable difficulty was always encountered when solvent precipitation was employed in an attempt to purify any of the fractions. The materials were hard to recrystallize and degradation took place most readily in many of the solvents as evidenced by color formation and decrease in yield of the substances finally obtained in the dry state.

Amberlite IR 4 offers no advantage over silver carbonate as a means of removing residual hydrogen chloride. Proline is practically eliminated as a product and only persists as an impurity in the hydroxyproline fractions. Whether this is due to racemization of the proline or catalyzed degradation due to a contribution of the resin or retention on the column are all open to question. It is highly probable that the proline was catalytically degraded in process by the contribution of the resin for the reasons already discussed. Purity and yields of hydroxyproline are also lower. However either silver carbonate or Amberlite IR 4 can be used for complete removal of residual hydrogen chloride.

The presence of ash and organic matter are problems which will have to be solved before the method will be of economic importance. Their eventual solution is quite within the realm of probability.

SUMMARY

1. A new method has been developed for the isolation of proline and hydroxyproline from collagen.
2. The procedure involves treating a collagen hydrolyzate with 0.3 N nitrous acid in the presence of an excess of 1 N hydrochloric acid at 60°C for 90 minutes, subsequently boiling off the excess liquid, extracting with concentrated hydrochloric acid, removal of residual hydrogen chloride, and finally absolute ethanol extraction to yield two crystalline moieties.
3. Three similar experiments, using silver carbonate to remove residual hydrogen chloride were carried out. Good yields were obtained in each case although the products were not pure.
4. Amberlite IR 4 offers no advantage over silver carbonate as a means of removing residual hydrogen chloride.

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