SEPARATION OF PROLINE AND HYDROXYPROLINE USING THIN LAYER CHROMATOGRAPHY

by

Derek Myhill

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[redacted]

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LEGENDS

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INTRODUCTION

Our present knowledge of the fine structure of the collagen fibril has come mainly from studies involving x-ray diffraction, electron microscopy and chemical examination of the amino acid composition and sequence, each of which revealed some unique features indicating that the collagen class of proteins is based on some plan of molecular architecture different from that of any other protein.

Structure of Collagen

A wide variety of investigations has established the fact that native collagen fibrils possess an axial repeat period of 640 Å, within which are many finer bands also visible in the electron microscope (2, 47). X-ray diffraction studies have indicated that the bands are due to alternating regions of strongly polar or nonpolar amino acids (3, 18). Since many of the bands are only 15 Å in width the regions could extend over relatively few amino acid residues. The work of Grassman et al. (15) has shown that this situation actually exists. If peptides isolated from tryptic digests of collagen were listed in order of increasing content of diamino plus dicarboxylic acids then this was also the order of decreasing content of proline plus hydroxyproline. Glycine was present in all peptides, usually in the amount of one-third of the total residues. Thus it seems probable that the sequence (- gly - pro - hypro -) postulated by Schroeder et al. (51) and actually isolated by Kroner et al. (24) accounts for a significant portion of the imino acids

in collagen.

This unusual pattern of amino acid distribution is extremely significant in view of the high content of glycine, proline and hydroxyproline found in collagens. In fact these three amino acids alone account for some two-thirds of the total residues in collagen. Only the collagens contain large amounts of hydroxyproline. Indeed it occurs elsewhere only in small amounts in elastin and enamel keratin and therefore hydroxyproline is unique to collagen for all practical purposes. The amino acid composition of collagen (11) is also remarkable for the low amounts of tyrosine and sulphur containing amino acids, and because of the unusual presence of a small amount of hydroxylysine which is again unique to collagen. Thus, determinations of the tyrosine content and the proline:hydroxyproline ratio (approximately 1.2) are widely used as a check on the purity of collagen samples.

The imino acids have a tertiary nitrogen in their peptide link and therefore the polypeptide chains cannot form single stranded helices, as do many proteins. Instead the collagen structure is based on triads of polypeptide chains held together by hydrogen bonds between different chains. The structure best accepted as accounting for the observed x-ray diffraction patterns has a polypeptide chain configuration similar to that found in polyglycine II (9, 45), which suggests that every third residue is glycine and is strongly supported by the work of Grassman already mentioned.

Biosynthesis of Collagen

(1) Hydroxylation of proline

Contrary to the general observation that proteins are built up from their constituent amino acids, Stetten (55) in 1949 made the fundamental observation that hydroxyproline of collagen is almost exclusively derived from proline. The only close parallel to this observation is that of hydroxylysine being derived from lysine (52), also found in collagen.

The importance of ascorbic acid in collagen metabolism has been established by numerous investigators, using morphological criteria (59). The results obtained by Stetten, and also the demonstration by Robertson and Schwartz (47) that the lack of collagen formation in ascorbic acid deficiency is due to inability to hydroxylate proline, suggested that a proline rich macromolecular precursor of collagen existed which subsequently became hydroxylated. In later work, Robertson et al. (46) analyzed proline and hydroxyproline isolated from collagen and also noncollagenous fractions in guinea pig granulomas after injection of C^{14}-proline. These results however were not consistent with the proline rich precursor theory.

An alternative suggested by the results of Gould and Woessner (14), in a study of the changes in proline, hydroxyproline and glycine distribution in granulation tissue of regenerating skin, was that the precursor of hydroxyproline might be an activated proline such as prolyl adenylate or a prolyl soluble RNA complex. The recent isolation of a sol-

uble RNA hydroxyproline, from the chick embryo by Manner and Gould (27), and from regenerating wound tissue by Jackson, Watkins and Winkler (unpublished results), is good evidence for this type of precursor.

This question of the exact location of the hydroxylation step along the path of collagen synthesis has provided considerable controversy. Gerber, Gerber and Altman (13) found that the specific activity of free hydroxyproline was greater than protein bound hydroxyproline in both skin and urine. However, Lindstedt and Prockop (26) could not confirm these findings. More recently Peterkofsky and Udenfriend (37) described the formation of hydroxyproline from labelled proline by a cell free system from chick embryo. Labelled hydroxyproline was detected only in the ribosomal fraction, presumably in peptide linkage although evidence of substantial concentrations of a soluble precursor was not obtained. Despite much work on the relationship of free hydroxyproline to bound hydroxyproline in various systems during the last few years this controversy still persists.

Since the inhibition of hydroxylation of proline in ascorbic acid deficiency was first described by Robertson, the problem of the mechanism of this hydroxylation has received considerable attention. Chvapil and Hurych (8) successfully demonstrated hydroxylation in a medium containing ethylene diamine tetraacetic acid disodium salt, fe^{++}, ascorbic acid, H_2O_2 and proline. The dependence of the hydroxylation process on ascorbic acid has also been demonstrated by

Stone and Meister (56) in studies on the synthesis of collagen hydroxyproline in carrageenin granuloma minced slices. In the absence of ascorbic acid, tritium from labelled proline was not found to accumulate in the water of the medium.

It is now known from studies with O^{18} that molecular oxygen rather than water is involved in the hydroxylation (44). By H^3:C^{14} retention analyses in studies using radioisotopes of proline it has also been found that only one hydrogen is lost from proline during the hydroxylation process (12).

Boucek et al. (7) have shown that in cell free systems the supernatant fraction, containing mitochondria and microsomes produced the greatest amount of hydroxyproline. Unfortunately when Hurych and Chvapil (20) then studied the effect of combinations of different subcellular fractions on the formation of hydroxyproline, a decreased hydroxylation activity was found in all samples compared to the controls which contained no biological fractions.

Thus it is not yet possible to speak of hydroxylation by a particular subcellular fraction since the results obtained by Chvapil's group make it probable that further metabolism of both proline and the newly formed hydroxyproline occurs on addition of the individual subcellular fractions to the medium. Only after inhibition of the enzymes responsible for this effect would it be possible to decide which subcellular fractions are able to convert proline to hydroxyproline.

(ii) Tropocollagen biosynthesis

In 1948, Orekhovitch et al. (32) reported the widespread occurrence of some soluble forms of collagen. During the next few years it was found possible to produce reversibly from collagen solutions the fibril having 640 Å periodicity and in addition several atypical forms of collagen. This work summarized by Schmitt, Gross and Highberger (49) suggested the existence of a unit of collagen capable of aggregating in different ways (48), later referred to as "tropocollagen" (16).

Study of the various forms of collagen indicated tropocollagen was a rodlike particle about 2800 Å in length. Confirmation of the existence of this hypothesized particle came when Boedtker and Doty (6), using ultracentrifugation to study the denaturation of collagen, prepared a monodisperse solution of a particle of molecular weight 345,000 having dimensions of 2800 Å by 14 Å. This macromolecule dissociates into two components, of molecular weights 80,000 and 160,000, which are usually referred to as α and β (the heavier). Doty and Nishihara (10) have since discovered that the β component at pH 12 gives rise to two units similar to the α component.

More recently, Piez, Lewis, Martin and Gross (39) demonstrated the presence of four major components in denatured skin collagen, by chromatography on carboxymethylcellulose. Two of the components had sedimentation behaviours similar to the original α component, whilst the other two were

similar to the original β component. This suggests that the collagen molecule contains three subunits of which two are α and one is β, the subunits presumably representing single chains extending the full length of the molecule. As the molecule matures the α chains cross-link intramolecularly in pairs to form β components, extractability of the collagen being directly related to the extent of cross-linking. A more mature form of collagen than the all α or β forms is γ tropocollagen (1), which consists of the three-stranded helix.

Much histological and electron optical evidence indicates that tropocollagen molecules, synthesized by the fibroblast, are secreted into the extracellular space where aggregation takes place. Tropocollagen molecules overlap from one row to the next, by one-quarter of their length, and all pointing in the same direction, which results in the 640 Å periodicity characteristic of the collagen fibril.

(iii) Delay in uptake of hydroxyproline into collagen

Studies on intact chick embryos (42) have demonstrated the conversion of proline to hydroxyproline and suggested that the microsomes are the initial site of collagen biosynthesis. Peterkofsky and Udenfriend (37) have also studied the hydroxylation process in a cell free system. In the presence of fortified microsomes from chick embryo homogenates, proline C^{14} is converted to radioactive hydroxyproline which is found in microsomal collagen like protein. The incorporation of proline and its conversion to peptide

bound hydroxyproline in microsomal, hot trichloroacetic acid extracts were followed for three hours. While proline incorporation began almost immediately and was maximal at 20 minutes, radioactivity was not detected in hydroxyproline until after 20 minutes.

This lag period may represent the time necessary for the hydroxylation of proline to occur and suggests it may be possible to study the hydroxylation process independent of protein formation.

(iv) Multiple peak uptake phenomenon

In most studies involving the uptake of isotopically labelled amino acids into collagen a single peak of activity was observed. The time of peak activity and the rate of fall to a minimum level have varied according to the conditions used in the experiment, but in general only one peak has been observed in the uptake curve. In several distinct experiments on the uptake of C^{14} glycine and H^3 glycine into collagen Bentley and Jackson (4) have observed a double peak in the uptake curve. This unexpected variation in the pattern of amino acid incorporation was detected because these workers studied much earlier and more frequent time points than had previously been reported. The significance of more than one peak of activity is not yet apparent since there is no evidence for the existence of two types of collagen in any particular tissue. Although purified skin collagen was counted as such after incorporation of radioactive glycine, the existence of two peaks could conceivably be due to the

presence of a noncollagenous impurity which is also taking up the glycine.

Use of Radioisotopes in the Study of Collagen

An important factor in radioisotope studies on collagen, emphasized by both the delay in incorporation of hydroxyproline into collagen and the multiple peak uptake phenomenon, is the amino acid to be employed as the isotopically labelled tracer. Until the last few years many studies on collagen utilized glycine as the tracer since this amino acid constitutes approximately one-third of the residues in collagen. However this necessitates extensive purification of the collagen and in any case isolation of glycine, usually as the dinitrophenol derivative (35), is a long and tedious way to determine the specific activity of collagen.

The recent availability of isotopically labelled proline has fulfilled the need for a more specific tracer than glycine. Since hydroxyproline, the imino acid unique to collagen, is not taken up for use in collagen synthesis when fed to growing animals, proline becomes the most specific tracer we can employ because it is incorporated into collagen as both proline and hydroxyproline. However C^{14} proline is expensive and also is not useful in many investigations on the hydroxylation problem since the specific activity is independent of the hydroxyl group. Very recently $3,4,H^{3}$ proline became available at a reasonable cost and is already widely used in studies on collagen and hydroxyproline biosynthesis.

It is therefore now possible to take full advantage of the fact that, in many tissues, for all practical purposes all protein bound hydroxyproline is in collagen. By removing free amino acids from a protein extract and isolating radioactive hydroxyproline from the total protein hydrolysate it would be possible to determine the specific activities of collagens without further purification. Comparison of the rates of incorporation of radioactive proline into collagen proline and hydroxyproline is another obvious approach in studies on collagen, however in this case purification of collagen is necessary in addition to separation of proline and hydroxyproline.

In view of the unusual findings in studies on collagen biosynthesis, and the fact that most of these investigations involve radioactive isotopes of proline, a reliable method of isolating pure samples of proline and hydroxyproline and determination of their specific activities is absolutely essential. Without such a method it will not be possible to investigate the complex relationships between free amino acid pool proline and collagen bound proline and hydroxyproline.

Survey of Previous Methods for Determination of Specific Activities of Proline and Hydroxyproline

As a preliminary to using the above approaches in studying the early stages of hydroxyproline and collagen biosynthesis it was necessary to examine the methods that are available for isolating pure samples of proline and hydroxy-

proline from protein hydrolysates. Specific methods of measuring the amount of proline or hydroxyproline in both isolated pure samples and protein hydrolysates were necessary and also a method of isolation that was capable of separating proline from hydroxyproline so that each could be determined simultaneously. Since the studies on collagen would involve determinations of specific radioactivity at different time points methods were also assessed in terms of rapidity and suitability for isotope work.

(i) Colorimetric determinations

Accurate measurement of amino acids may be carried out by a variety of photometric ninhydrin methods. However, the poor colour yield in the reaction of ninhydrin with imino acids makes such methods low in sensitivity, particularly in the case of hydroxyproline. Piez et al. (38) described a fairly simple procedure for the measurement of cyclic imino acids, which employs ninhydrin in glacial acetic acid. The colour reaction was carried out at room temperature and absorbance read at 350 mμ. Whereas hydroxyproline reached maximal colour in 130-140 minutes, proline colour development took even longer, 280 minutes. In the case of proline the reaction could be speeded up by performing the reaction in a boiling water bath and reading the colour at 40 minutes.

Troll and Lindsley (57) modified the ninhydrin reaction in several ways to allow accurate measurement of proline in a protein hydrolysate. These workers showed that interfering substances such as lysine and ornithine could be removed from

solution at pH 4.0 by "permutit according to Folin" ion exchange resin. In addition to this modification, the final coloured product was extracted into a smaller volume of benzene and this phase read in a colorimeter at 515 mμ. By these improvements in the method accurate measurement of solutions of proline containing 1-5 μg/ml were possible.

This modification by Troll and Lindsley was based on the belief that the only amino acids interfering with the method were ornithine, citrulline, lysine, hydroxylysine and cysteine. However Messer (28) has recently shown that the method is subject to interference by almost all the common amino acids and by some di and tripeptides when they are present at concentrations 10 or 20 times that of proline. A simple modification involving addition of glycine to the solution to be analyzed eliminates interference by 10 of the amino acids and increases the sensitivity of the method by 50%.

Several modifications of the isatin colour reactions with proline or hydroxyproline (33, 34) allow very specific determinations to be performed on filter paper chromatograms or on the eluted colour product. However these methods were designed for use with pure solutions of imino acids and would certainly not allow measurements on hydrolysates. Reference will later be made to the use of one such staining procedure in qualitative examination of chromatographed imino acids, however these methods have little value in routine quantitation of proline and hydroxyproline.

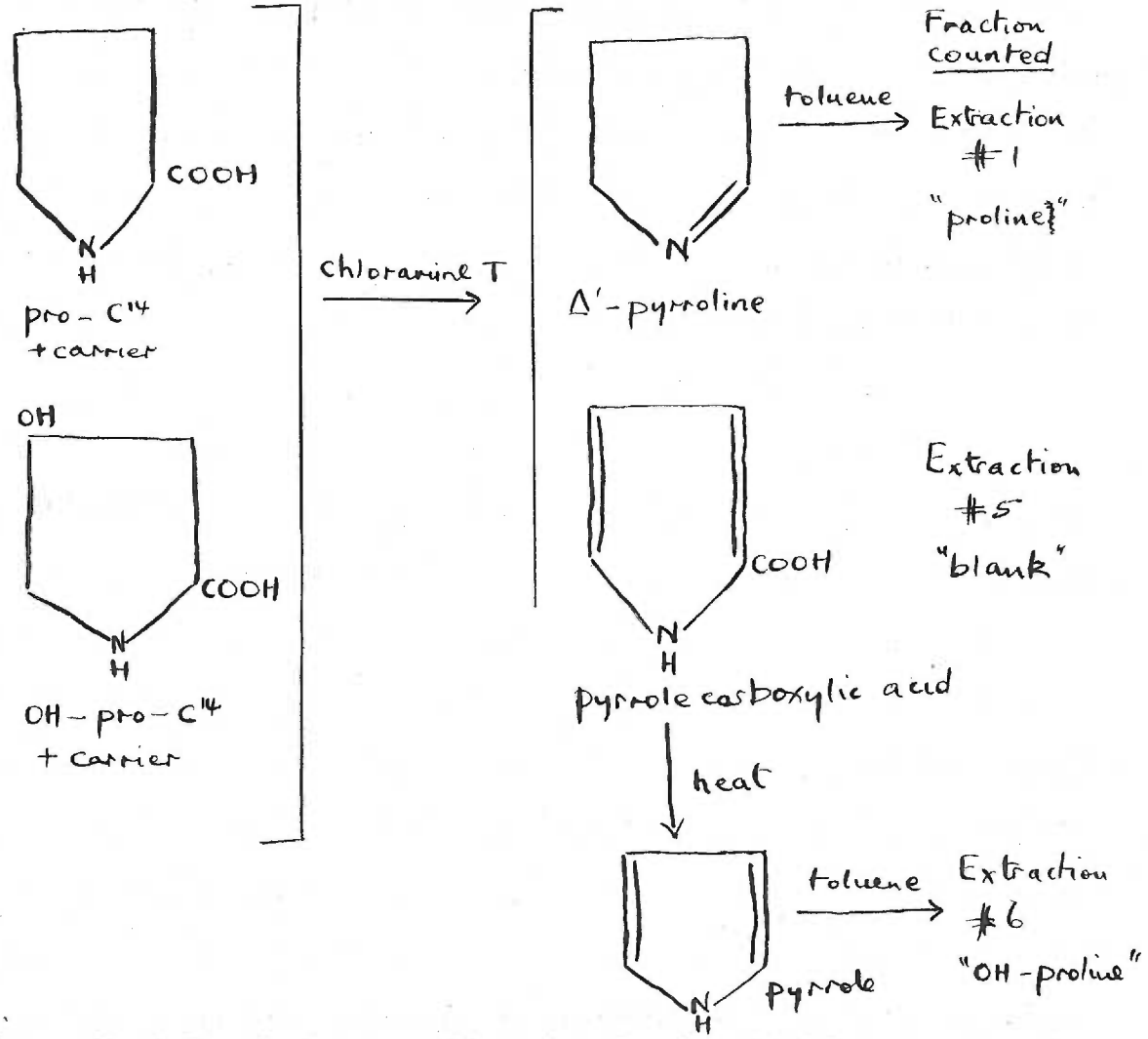
As previously mentioned the ninhydrin reaction is unsuitable for measurement of hydroxyproline because of the very low colour yield. Several methods for the quantitative analysis of hydroxyproline have involved oxidation of the imino acid to pyrrole and then condensation of the pyrrole with p-dimethylaminobenzaldehyde to form a chromophore suitable for spectrophotometry (17, 31, 41). In a recently described method for hydroxyproline assay Prockop and Udenfriend (43) extracted the pyrrole into toluene to separate it from interfering substances and to insure specificity of the colour reaction.

Since toluene solutions are frequently used as the medium in which scintillation counting is carried out, this extraction of pyrrole into toluene makes possible both colorimetric measurement of the amount of hydroxyproline and radioactivity measurements on the final toluene solution containing the pyrrole. This cuts out several of the usual steps in the process of radioactivity measurement by liquid scintillation counting, viz., drying down an aqueous solution of hydroxyproline, desiccation and then dissolution in hyamine hydroxide followed by addition of the toluene containing the phosphor.

In view of these possibilities Prockop et al. (41, 36) have modified their method several times so that at the present time their method is the best available for measuring the specific activity of labelled hydroxyproline in biological materials. One of the most recent of these modifi-

cations allows the simultaneous measurement of the radioactivity of proline and hydroxyproline in a single sample, which is a feature often to be desired.

An outline of the Prockop procedure is shown below.



The method for hydroxyproline involved oxidation to pyrrole by chloramine T. Because the initial oxidation products of hydroxyproline were not extractable from alkaline solutions by chloroform or toluene, interfering radioactive materials were removed by several such extractions before the pyrrole was formed. Since proline is also oxidized by chloramine T

and its oxidation product is soluble in toluene the following procedure is used. The product of proline oxidation is extracted into toluene and then several more extractions are carried out to remove traces of this compound remaining in solution. Upon heating the extracted reaction mixture the hydroxyproline intermediate oxidation product is then converted to pyrrole which is extracted into toluene. In order to ensure consistent recoveries relatively large amounts of carrier proline and hydroxyproline are added. Recovery of carrier proline is determined by comparison with a radioactive standard; the recovery of carrier hydroxyproline is determined colorimetrically. In this way colorimetric measurement and scintillation counting on the toluene solutions containing the various oxidation products of the imino acids allow the radioactivities of C^{14} proline and C^{14} hydroxyproline to be measured in a single aliquot.

Although this method offers many advantages over the time consuming paper or column chromatographic separations of these imino acids it also has many undesirable features. The method is complex and it is conceivable that the intermediates in the oxidation process have been inadequately characterized. The quantitation of the method is poor since the relatively large amounts of carrier proline and hydroxyproline may preclude investigation of fractions having a low specific activity. Also, in experiments involving H^3 proline a loss of 50% of the tritium is inherent in the method due to the oxidation step. Despite these disadvantages it should be

stressed that this is at present the most successful method of separating simultaneously proline and hydroxyproline in multiple samples of biological materials available.

(ii) Separation of imino acids

(a) Column chromatography

Several methods of separating amino acids by column chromatography have been described. In 1949, Moore and Stein (29) achieved separation of a number of amino acids on starch columns using organic solvent mixtures. Two years later these workers reported a procedure for the chromatographic separation of mixtures of amino acids by gradient elution analysis on Dowex 50 ion exchange resin (30). However it was not easy to obtain pure samples of proline and hydroxyproline by this procedure. More recently Levine (25) employed the nitrous acid reaction as a preliminary step to isolation of proline and hydroxyproline from protein hydrolysates. Isolated nitroso imino acids were regenerated to imino acids by hydrolysis with 6 N HCl and then separated by gradient elution with dilute HCl on a column of Dowex 50 resin, in relatively high yields.

Although the use of nitrous acid to destroy the amino acids in a protein hydrolysate was a useful approach which has since been used in several methods, Levine's method suffers from the general disadvantages of employing column chromatography for this purpose. This type of chromatography is unsuitable for multiple sample work and can easily become very time consuming unless it can be automated, which

would be somewhat expensive. Although separation of amino acids this way has become routine and also automated, because hydroxyproline occurs only in collagenous proteins many of these methods were reported without any mention of the position of hydroxyproline in the system.

(b) Filter paper chromatography

A number of workers (34) have used filter paper chromatography methods to separate proline and hydroxyproline. However, none of these methods was impressive since preliminary desalting was usually involved and two dimensional separations often required. Even the preliminary use of nitrous acid treatment does not solve these problems since preliminary desalting is still needed and although a single dimensional separation is then possible the amounts which may be separated are not impressive.

A major difficulty in this type of separation is that although reliable methods exist for the quantitative photometric determination of imino acids on the sprayed chromatogram or on the eluted colour reaction products, such highly coloured compounds show considerable self-absorption when one tries to measure their radioactivity by scintillation counting.

Most workers using filter paper chromatography employ the nitrous acid reaction followed by desalting on ion exchange resin as preparatory steps and therefore their procedure is probably as time consuming as methods performing the actual chromatography on the ion exchange column itself.

Thin Layer Chromatography

A method of chromatography which has never been applied to the separation of imino acids is thin layer chromatography, a new method of adsorption and partition chromatography on a micro scale, which supplements the previously known methods of column, paper and ion exchange chromatography. In operation a glass plate is coated with a thin layer of adsorbent which adheres to the plate. The layer represents an "open" column. The sample is spotted on this coating as a solution and is developed in a tank containing a small amount of the appropriate solvent, usually by the ascending technique. Only short travel distances (approximately 10-15 cm) are required.

During the last few years equipment for thin layer chromatography has become commercially available and the method, which was originally designed for separation of oils and lipids, has been applied to separation of many organic compounds (60).

The main features of Stahl's rapid thin layer technique (54) are the simplicity of method and apparatus and the wide quantitative range handled. This suggests thin layer chromatography may be the method of choice in separation of proline and hydroxyproline.

Design of a Rapid Method of Isolating Pure Samples of Proline and Hydroxyproline

It was decided to investigate the suitability of thin layer chromatography for separation of large amounts of pro-

line and hydroxyproline rapidly and from multiple samples. In accordance with filter paper chromatography findings, butanol/acetic acid/water solvent (63:27:10 v/v) was used, with cellulose powder as the adsorbent (5).

As a preliminary step protein hydrolysates would be nitrous acid treated and the amino acid deamination products removed by ether extractions as in the work of Levine. If possible thin layer chromatography would be performed directly on the nitrous acid treated residue after extraction with ether. The regeneration and desalting steps would be omitted provided the thin layers were capable of separating the imino acids from a solution containing some residual salt introduced with the nitrous acid reagent.

Having determined the suitability of this system for isolating the imino acids as pure samples from protein hydrolysates, by examination of recoveries at each stage and purity of eluted samples, etc., the validity of using the method would have to be confirmed. Two methods of checking the validity were decided on, one involving a simple mixture of radioactive proline and nonradioactive hydroxyproline, the other being an application of the method to study of a biological system.

Applications of the Method, in order to confirm:

(i) Validity of the method

After chromatography and elution of various mixtures of proline and hydroxyproline of which only the proline was radioactive the radioactivity found in each sample was measured

and corrected to d.p.m., by the use of internal standards. As distinct from straightforward expression of % contamination the results were considered in terms of changes in the specific radioactivity of the proline, and more important, the apparent specific radioactivity of the hydroxyproline. Significance of these results was viewed in relation to the range of specific activities of proline and hydroxyproline usually encountered in radioisotope studies on collagen.

(ii) The multiple peak uptake curve of collagen

Before undertaking further investigations of the nature of the double peaks found in the uptake curves of collagen it was necessary to confirm the multiple peak phenomenon using the specific tracer H^3 proline. This would exclude the possibility that contamination could have been responsible for the results obtained by Bentley and Jackson who counted purified skin collagen after incorporation of labelled glycines. This confirmation experiment, if in agreement with the findings in several distinct experiments by these workers, would also be a valuable confirmation of the thin layer chromatography method.

The collagen fraction studied by Bentley and Jackson was that soluble in neutral salt solution. After a persistent controversy about the soluble precursors of collagen Jackson and Bentley (21) investigated the significance of these extractable collagens and showed that a whole spectrum of collagen aggregates of varying degrees of cross linkage exist in developing connective tissues. The frac-

tion soluble in 0.14 M NaCl, which had previously been shown to contain tropocollagen molecules (22), contained the most recently synthesized collagen. It was this newly formed collagen, extracted from guinea pig skins, that was examined by Bentley and Jackson when the multiple peak phenomenon was observed. The same fraction was used in the study presented here as confirmation of the thin layer chromatography method for separation of proline and hydroxyproline.

In the skin experiment described the thin layer chromatography method has been used to isolate pure samples of radioactive protein bound hydroxyproline in hydrolysates from relatively crude neutral salt extracts of skin, at various time intervals after injection of 3,4,H^3 proline.

EXPERIMENTAL

Apparatus

Desaga-Brinkman equipment was used for thin layer chromatography. The glass plates used were 8"x8" and the applicator was nonadjustable giving a standard layer of 250 μ thickness.

All radioactivity measurements were carried out using a Packard Tri-Carb model 314 automatic scintillation spectrometer.

Materials

Nitrous acid was prepared by adding 3 ml of 40% sodium nitrite solution to 10 ml of concentrated HCl, in an ice bath. After salt had settled the supernatant was removed.

Proline and hydroxyproline standards were purified by chromatography on Dowex 50 x 8 (H^+) ion exchange resin with an elution gradient from 1.5 N HCl to 4 N HCl.

Benzoic-C^{14}-acid; 2,C^{14}-L-glycine; 3,4,H^3-L-proline; and uniformly labelled C^{14}-L-proline and H^3-toluene, were all obtained from New England Nuclear Corporation.

Scintillator solution: reagent grade toluene containing 0.4% 2,5-diphenyl-oxazole (PPO) and 0.01% 2,2-p-phenylene-bis (5-phenyloxazole) (POPOP).

Thin layer chromatography

Forty gram of cellulose powder (without $CaSO_4$ binding agent) was suspended in 200 ml of distilled water and spread on the plates using the applicator. These quantities were found suitable for filling the applicator and getting an

even flow of the right thickness. Plates were then air dried and stored in a desiccator. Unknowns and marker spots, containing proline and hydroxyproline, were applied to the thin plates in 10-20 λ volumes containing 100-500 μ gm of each imino acid. After ascending chromatography for 5 hours in butanol/acetic acid/water solvent (63/27/10 v/v), plates were dried at 110° C for 15 minutes. The marker spots were then sprayed with 0.2% ninhydrin in acetone and the plates heated at 110° C for 15 minutes to reveal the positions of proline and hydroxyproline.

Elution of proline and hydroxyproline

The positions of the unknowns were outlined in pencil, using the stained marker spots as a reference, and the cellulose within the pencilled areas cut out with a razor blade and collected in a tube. Imino acids were eluted from the cellulose by two successive extractions with boiling distilled water, using 10 ml of water each time. Eluates were pooled, filtered and made up to 25 ml with distilled water. Determinations of proline and hydroxyproline and also radioactivity measurements were carried out on this eluate.

Isolation of imino acids

One to ten mgm of protein was hydrolyzed in 6 N HCl for 3½ hours at 138° C in sealed tubes. Hydrolysates were decolorized with activated charcoal, filtered and evaporated to dryness in a steam bath. Excess nitrous acid reagent (1 ml/mgm of amino acids) was added to the hydrolysate residue, the mixture allowed to stand at room temperature for 10

minutes and then heated at 138° C, until the colour was only faintly yellow. The acid mixture was evaporated off in a steam bath and the residue extracted with 5 ml of ether to remove deamination products. Under these conditions imino acids are present unchanged or as unstable n-nitroso derivatives. After ether extraction the residue is taken up in 0.1 ml of 70% ethanol, which leaves much of the salt behind. Proline and hydroxyproline could then be separated from this solution by thin layer chromatography as described.

Determination of specific activities

Proline determinations were by the Troll and Lindsley modification of the Chinard ninhydrin reaction (56) and hydroxyproline determinations by the method of Woessner (57). A known volume of eluate was also evaporated to dryness in a glass vial, the residue dissolved in one ml of X-10 Hyamine hydroxide, 10 ml of scintillator solution added and radioactivity measured by liquid scintillation counting. The counting efficiency was determined by the use of C^{14}-benzoic acid or H^3 toluene as internal standard. Counts per minute (c.p.m.) were then corrected to disintegrations per minute (d.p.m.) and specific activities expressed as d.p.m./μMole of proline or hydroxyproline.

Application of the method: in vivo incorporation of H^3 proline into collagen hydroxyproline

Thirty-three young female guinea pigs were decapitated in groups of three at regular intervals between 2 and 8 hours after intraperitoneal injection of 45 μc of 3,4,H^3-L-

proline/animal. Skin was removed from each group, cooled, and crushed after being frozen in liquid nitrogen. A weighed amount of skin was extracted three times with 0.1$\frac{1}{4}$ M NaCl solution buffered to pH 7.5 with phosphate. All extractions were carried out at 2° C for 2$\frac{1}{4}$ hours. Extracts were pooled and filtered and an aliquot of each removed for determination of total collagen extracted. The aliquots were dialyzed three times, 2$\frac{1}{4}$ hours at a time, against 0.01 M acetic acid, dried, hydrolyzed and collagen determined by measurement of hydroxyproline in the hydrolysate. The rest of this neutral salt extract was precipitated with solid NaCl to a final concentration of 20%, followed by resolution in 0.01 M acetic acid and dialysis three times, for 2$\frac{1}{4}$ hours each time, against this same solution. This single precipitation and dialysis were necessary to remove the free amino acids present in the extract. The final collagen solutions were dried down in a steam bath, the residues hydrolyzed and hydroxyproline isolated as described above. The specific activity of protein bound hydroxyproline was obtained for all extracts and the incorporation of H^3 proline into collagen hydroxyproline thus determined for the particular time points studied.

Figure 1. Quantitative chromatographic separation of equal amounts of proline and hydroxyproline in mixtures containing up to one mgm total imino acid.

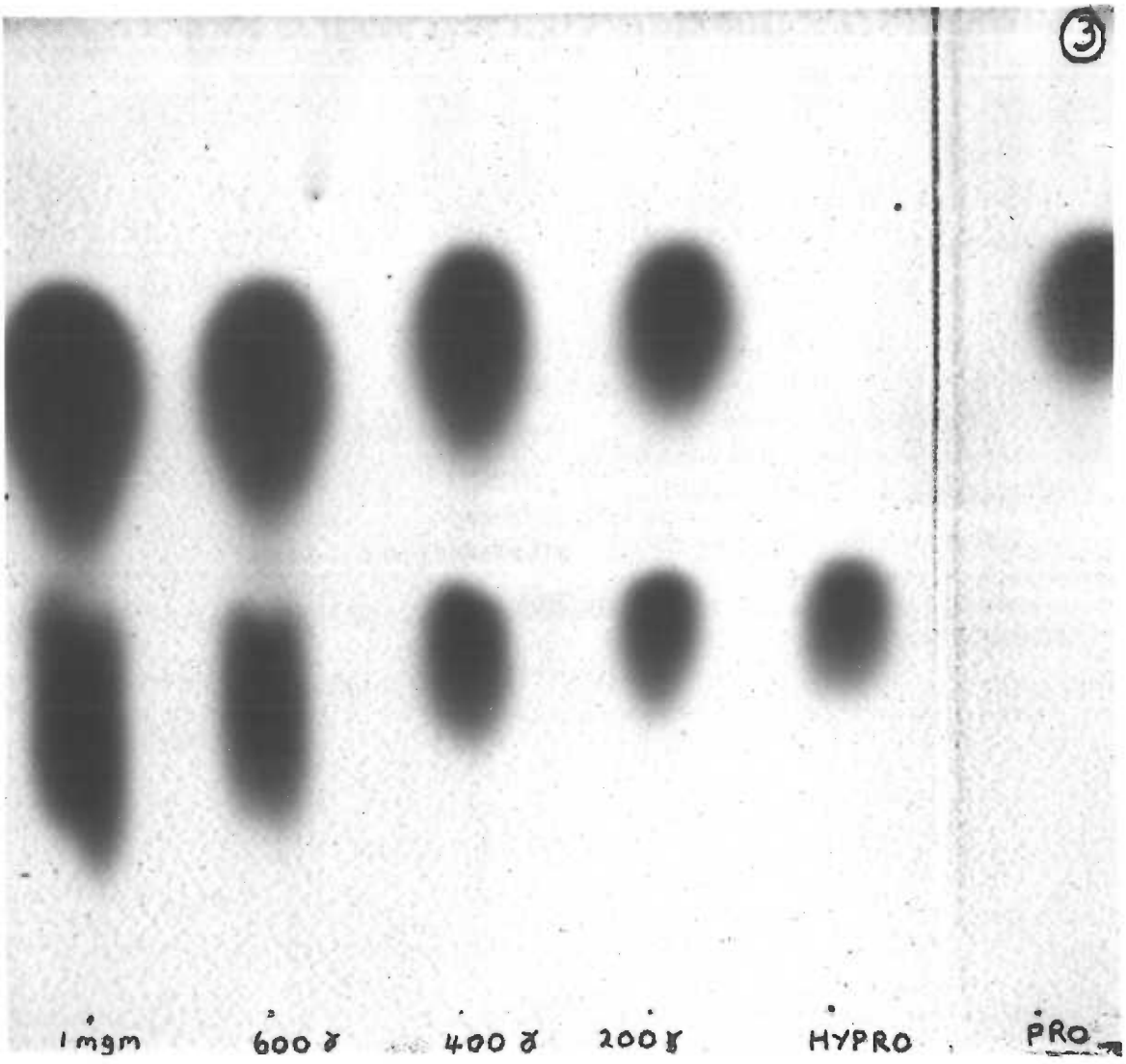

3
1 mgm
600 γ
400 γ
200 γ
HYPRO
PRO

Figure 2. Area to be eluted for maximum purity.

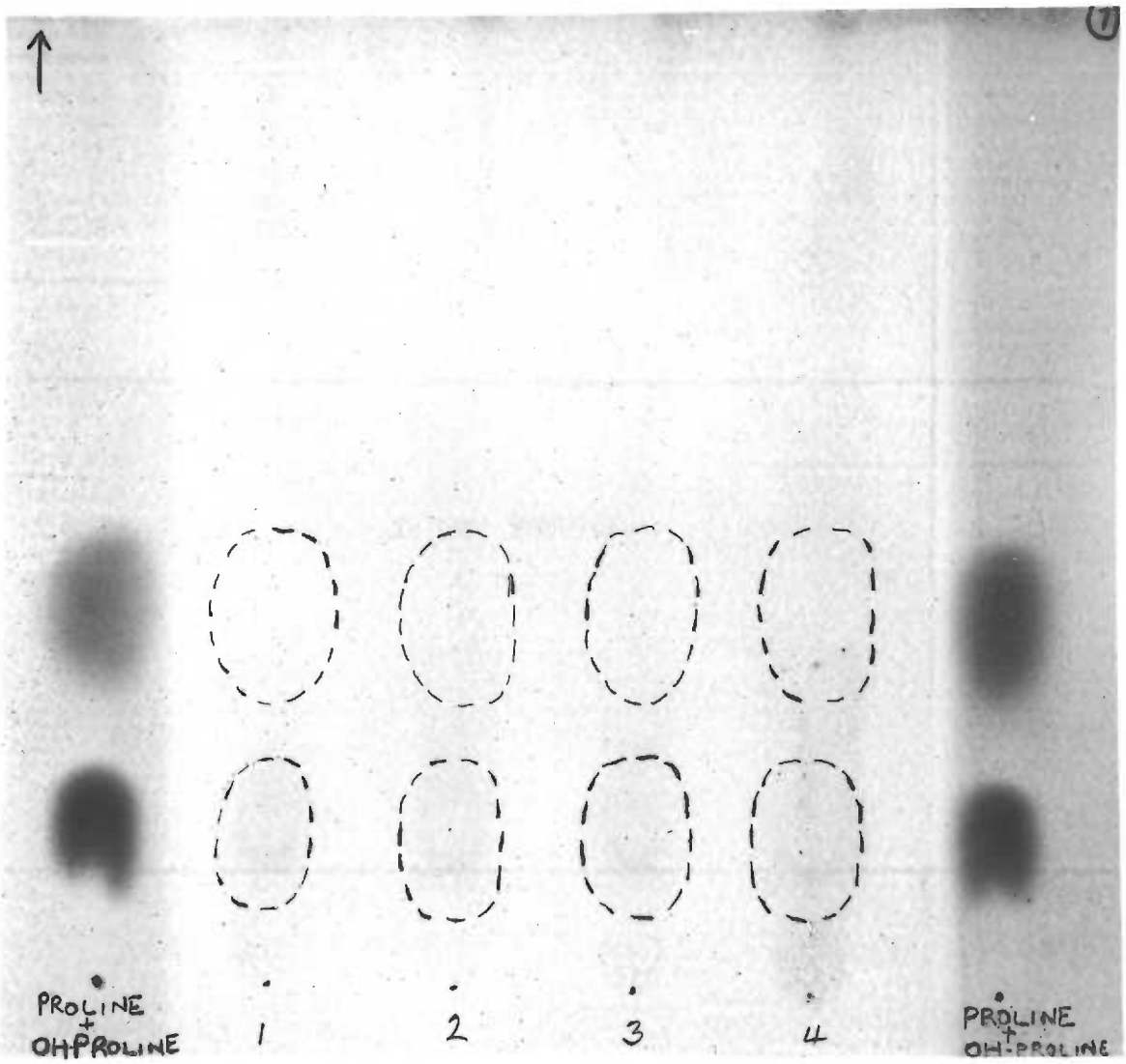

PROLINE + OH-PROLINE
1
2
3
4
PROLINE + OH-PROLINE

TABLE I

Recovery of Imino Acids by Elution

Initial μg Hydroxyproline	# of Pooled Elutions (1)	μg Eluted	% Recovery
200	1	118	59
	2	128	64
	3	131	65
	4	131	65
200	4	122	61
200	4	127	63.5

(1) Each elution consisted of 10 ml of boiling distilled water.

Figure 3. Relationship of apparent position to recovery by elution. The amount of proline chromatographed was 200 μg in each case. Areas depicted by A, B, C and D on the chromatogram were cut out and eluted twice with 10 ml distilled water. Elution recoveries are shown in Table II.

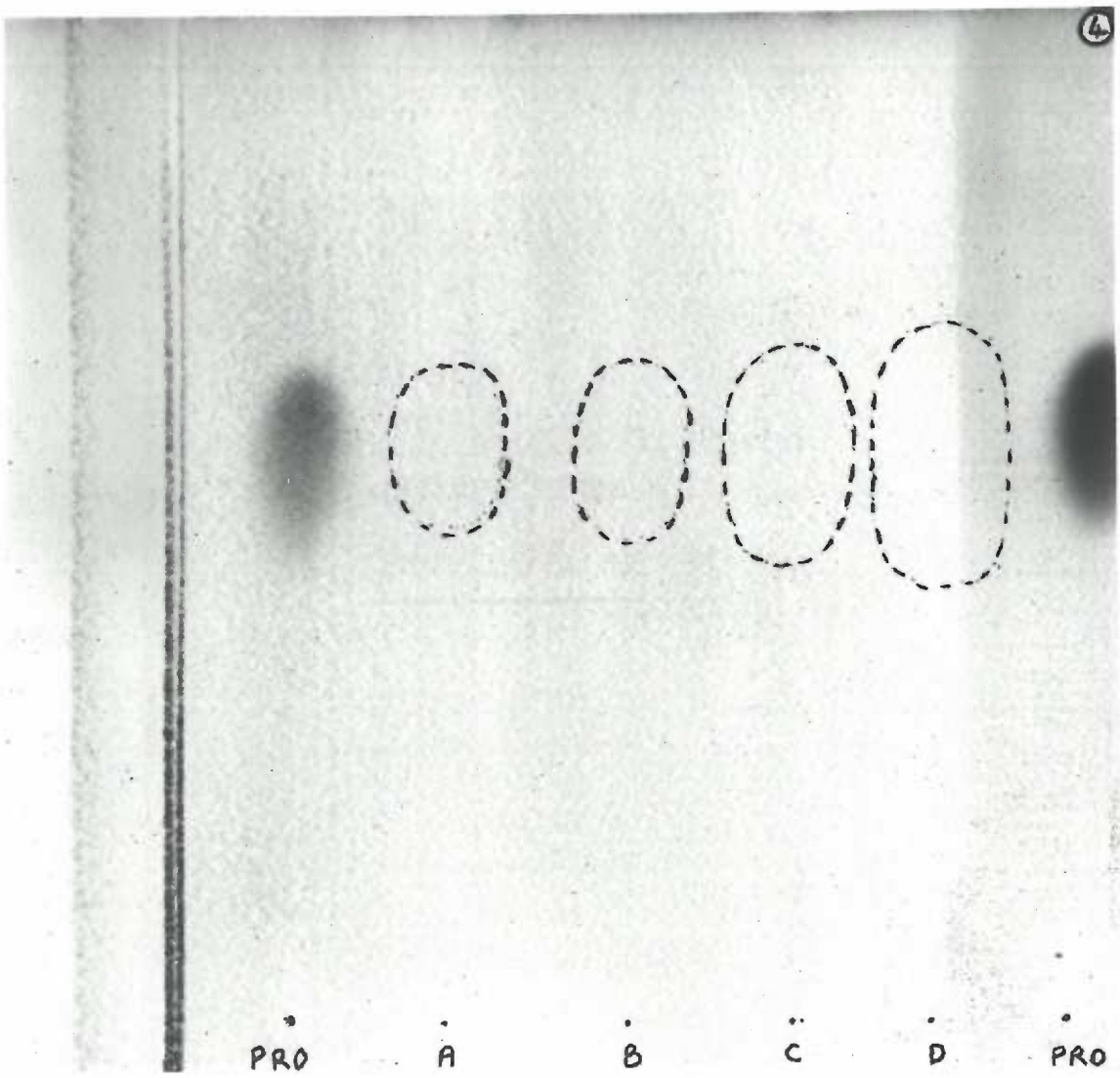

4
PRO
A
B
C
D
PRO

TABLE II

Relationship of Apparent Position to Recovery by Elution

Area Eluted (1)	µg Proline Eluted (2)	% Recovery (3)
A	126	63
B	142	71
C	178	89
D	168	84

(1) See Figure 3 for description of relative areas denoted by A, B, C and D.

(2) Two pooled elutions used in each case.

(3) In each case 200 µg proline was chromatographed.

TABLE III

Purity of Eluted Imino Acids

Initial μg		d.p.m.	% Contami-	d.p.m.
Proline	Hydroxyproline	Eluted (1)	nation	Mole
91		6,888		8,694
	105	57	0.8	71
182		13,817		8,740
	210	140	1.1	88
273		20,104		8,510
	315	284	1.4	118

(1) Two elutions with 10 ml boiling distilled water.

TABLE IV

R_f Values of Imino Acids

	R_f	Color with Ninhydrin
L-Proline	0.70	Yellow
N-Nitroso-L-Proline	0.64	Reddish-yellow
L-Hydroxyproline	0.36	Yellow
N-Nitroso-L-Hydroxyproline	0.35	Orange

Figure 4. Effect of nitrous acid treatment upon a hydrolysate of gelatin.

GT: One mgm of gelatin hydrolysate.

GTN: One mgm of gelatin hydrolysate after nitrous acid treatment.

GTNE: As GTN but also ether extracted.

N-PRO, N-HYPRO: Marker spots of nitrous acid treated proline and hydroxyproline respectively.

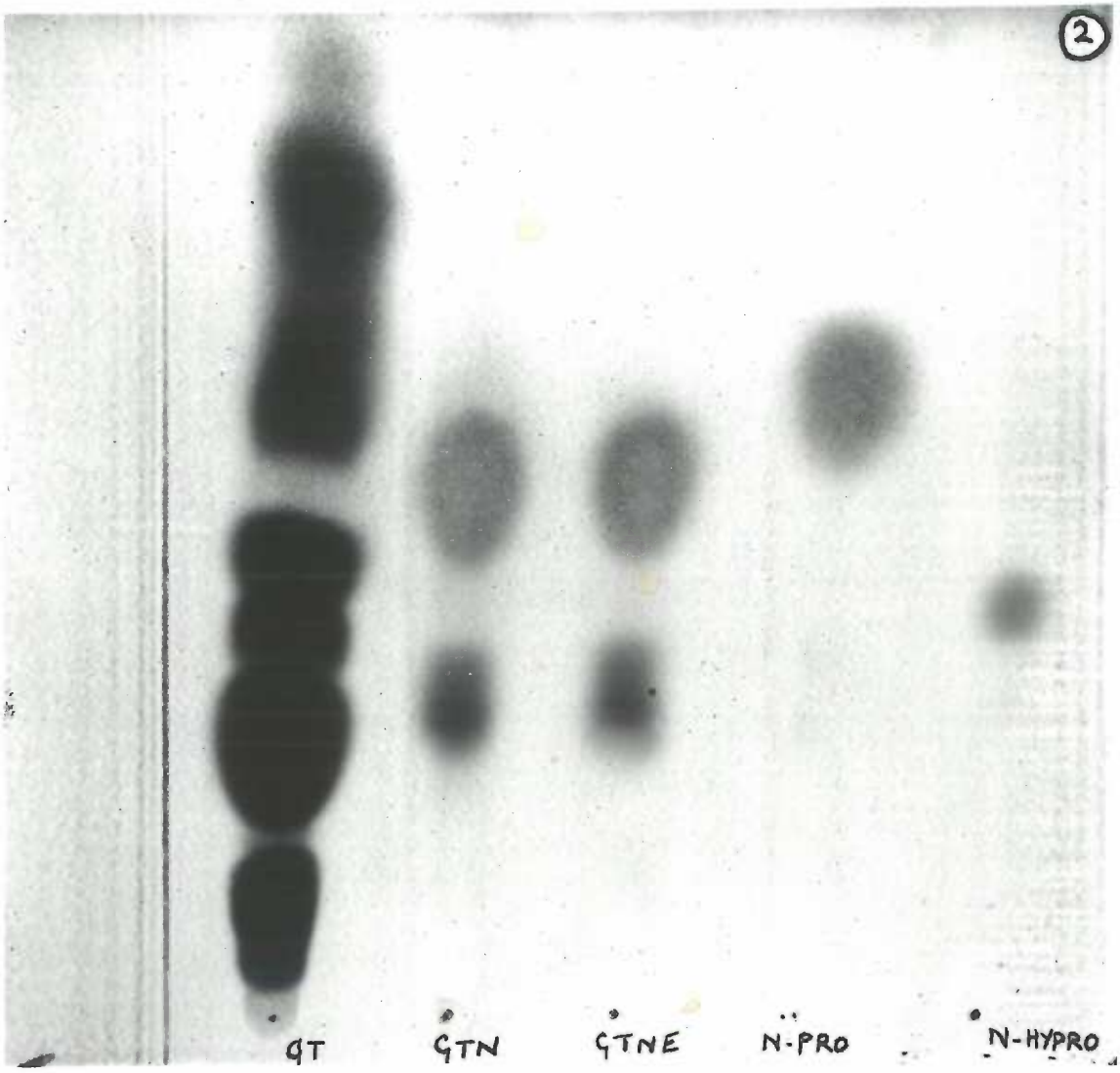

2
GT
GTN
GTNE
N-PRO
N-HYPRO

TABLE V

Removal of Amino Acid Derivatives by Ether Extractions
(results as disintegrations per minute)

Sample (1)	Untreated	Nitrous Acid Treated and Ether Extracted (2)	% Lost
C^{14}-Proline	516	355	31
	581	387	33
C^{14}-Glycine	21,181	105	99.5
	18,027	222	99

(1) Amounts used: proline 80 μg including cold proline,
glycine 209 μg including cold glycine.

(2) Three successive ether extractions of 5 ml were used on each sample.

TABLE VI

Overall Recovery of Imino Acids

	Untreated (μg)	Treated (1) (μg)	Eluted (2) (μg)	% Overall Recovery
Proline	200	172	125	63
Hydroxyproline	200	166	117	58

(1) Nitrous acid treatment followed by a single extraction with 5 ml of ether.

(2) Two elutions with 10 ml of boiling distilled water.

Figure 5. Specific activity of radioactive protein bound hydroxyproline in 0.14 M NaCl extracts of guinea pig skin between 2 and 8 hours after intraperitoneal injection of 45 μc of 3,4,H^3 proline. Calculations of d.p.m./μ Mole hydroxyproline are as shown in Table VII below.

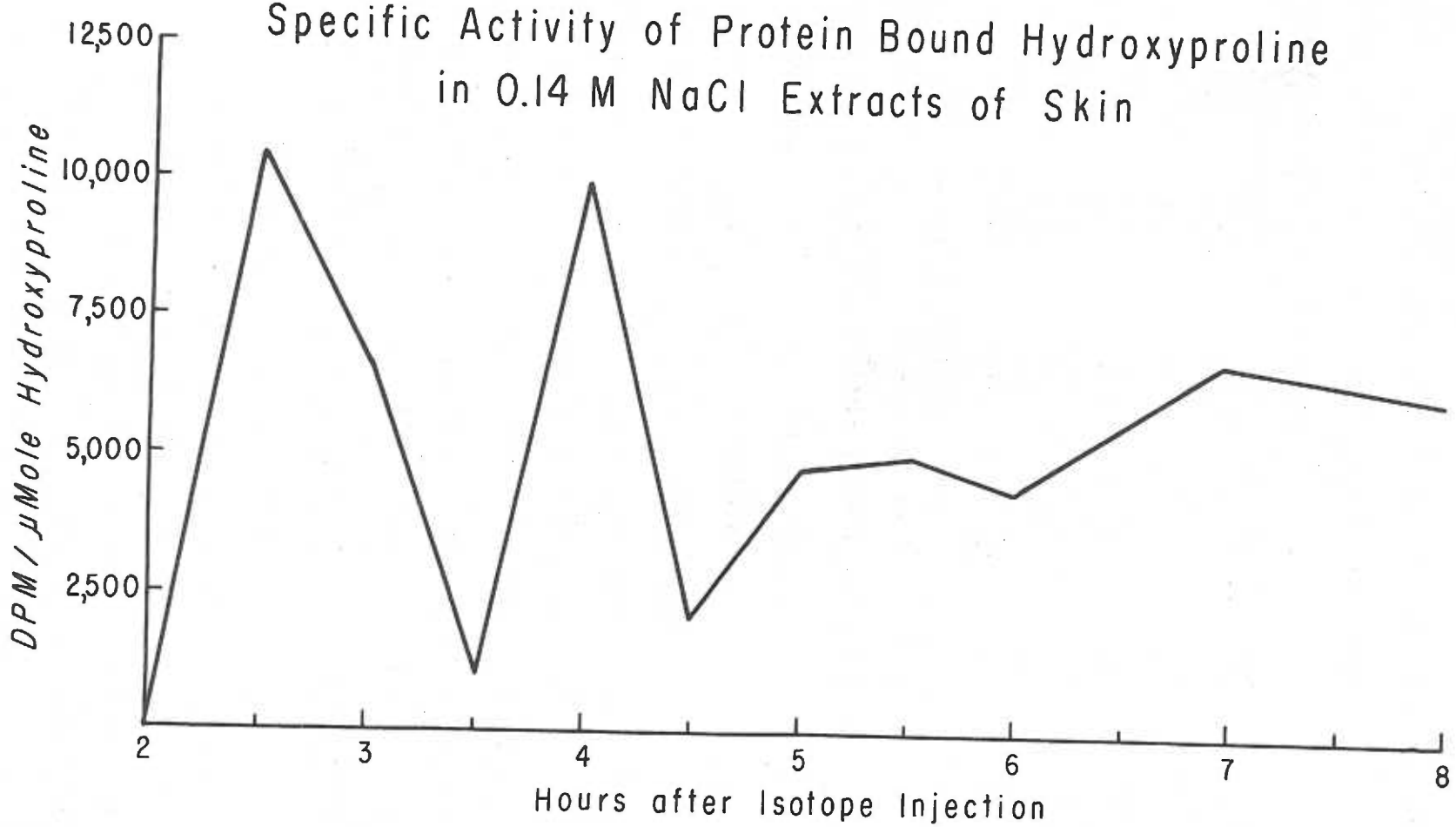
TABLE VII. Determination of the Specific Activities Shown in Figure 5. Results of the skin experiment are expressed as d.p.m./μMole of hydroxyproline, which was calculated as shown below. Correction of c.p.m. to d.p.m. was necessary since self-absorption differs from sample to sample, making comparisons of c.p.m./μMole of little value in this type of experiment. One ml of H^3 toluene, having a known d.p.m., was added to each vial and the samples recounted. The efficiency of counting was then calculated from the increase in c.p.m. and the sample d.p.m. then determined.

Time Point (hours)	c.p.m. (1)	Increase in c.p.m. (2)	% Efficiency	d.p.m. (3)	μgm Hydroxyproline Counted	d.p.m./μ Mole
2	9	780	6.79	177	80	262
2½	167	445	3.89	5,573	72	10,480
3	166	469	4.09	5,404	106	6,681
3½	28	789	6.88	542	64	1,048
4	189	521	4.54	5,549	72	10,087
4½	87	836	7.29	1,590	97	2,096
5	172	733	6.39	3,588	96	4,847
5½	111	497	4.33	3,417	88	5,109
6	375	813	7.09	7,052	205	4,454
7	366	537	4.68	10,426	195	6,943
8	243	905	7.89	4,105	84	6,288

(1) After subtraction of background c.p.m.

(2) After addition of 11,475 d.p.m. of H^3 toluene.

(3) Corrected for the 25% loss of H^3 during in vivo hydroxylation of proline.


Specific Activity of Protein Bound Hydroxyproline
in 0.14 M NaCl Extracts of Skin
DPM / µMole Hydroxyproline
12,500
10,000
7,500
5,000
2,500
2
3
4
5
6
7
8
Hours after Isotope Injection

RESULTS AND DISCUSSION

Thin layer chromatography of imino acids

Although separation of proline and hydroxyproline is adequate using this system, it is advisable to include a reference mixture on each chromatogram since R_f values on thin layers are not as reproducible as on filter paper. Each sample should preferably be applied to a thin plate in duplicate, using the same volume each time, and one of these stained with ninhydrin, in order to determine the exact positions of proline and hydroxyproline after separation. Three or four unknowns may be separated on each plate even when samples are chromatographed in duplicate.

Mixtures containing up to 500 μg of each imino acid applied in up to 50 λ of 70% ethanol can be separated as shown in Figure 1. The usual working range on the plates involves 100-250 μg of each imino acid, separation of which is quite good. Should larger quantities of imino acids be required, use of the variable thickness applicator may increase the amounts separated by a large factor.

Recovery of imino acids by elution

Since more than 90% of the total imino acid recovered was found in the first two elutions with distilled water, as is shown in Table I, only two elutions, each of 10 ml, were considered necessary in subsequent work. Recovery from the chromatograms was initially only in the region of 60%, however this was increased to 70% by taking a slightly larger area for elution, as depicted in Figure 3.

The low colour yield of the ninhydrin reaction with imino acids is probably partly responsible for this situation. An alternative staining procedure with greater sensitivity involves spraying with 0.2% isatin in acetone and heating at 75° C, which reveals both imino acids as blue spots (52). If the chromatogram is then sprayed again, this time with a solution of 1 g of p-dimethylaminobenzaldehyde, 90 ml of acetone and 10 ml of concentrated HCl the hydroxyproline spot turns a purple red colour (23). However, since 100-200 μg of each imino acid is easily obtainable with a reasonable degree of purity, as seen in Table III, exact recovery is not too important and therefore in routine use ninhydrin staining is usually satisfactory.

The quantitative relationship between the area of cellulose eluted and recovery is shown in Table II. Whereas the recovery can be increased enormously by increasing the area it also presumably increases contamination. In subsequent work only a small increase in the area eluted has been commonly used which has resulted in recoveries in the region of 70% (as seen in Table VI). In view of the quantities handled by this method this recovery was considered quite adequate and no further effort was made to increase the elution recovery beyond this level.

Purity of eluted samples

Table III shows the results of an experiment which was designed to assess the validity of using the eluted samples to determine the specific activity of free or protein bound

hydroxyproline. To a mixture of proline and hydroxyproline was added a negligible amount of H^3 proline of high specific activity. Increasing amounts of this solution containing 100-300 g of each imino acid were chromatographed and the eluted samples analyzed to determine the apparent specific radioactivity of proline and hydroxyproline over this range of separation. Colorimetric measurements of the amount of contamination had indicated little or no contamination in eluted samples, however in the type of experiments required for study of collagen biosynthesis even a minimal amount of contamination of high radioactivity could lead to a very misleading set of results. As can be seen in Table III the amount of contamination is of a low order, particularly if one considers the short length of chromatogram and the rapidity of the method employed. However considerably more significant than this is the fact that the specific activity of the proline stays essentially the same and the effect of this small amount of contamination on the specific radioactivity of the hydroxyproline is extremely small.

The specific activities found for hydroxyproline are less than would result from 1 d.p.m./ μg which is near the lower limits of scintillation counting anyway. To illustrate this point the lowest specific activity recorded in the skin experiment involving uptake of H^3 proline into collagen hydroxyproline, results of which may be seen in Table VII, was 262 (i.e., 2 d.p.m./ μg). This figure in relation to the 1,000-11,000 d.p.m/ μMole values obtained for the rest of the

time points is essentially zero. In addition to this, the efficiency of counting as much as 100 μg of radioactive hydroxyproline is usually only in the region of 5-7% which makes the actual c.p.m. recorded extremely close to background anyway. Thus if the specific activity is not more than 2 d.p.m./μg, in many instances this amount of contamination would not even be detected by scintillation counting.

The results presented in Table III therefore clearly indicate that separation of the imino acids by the thin layer chromatography method is suitable for studies on collagen that involve the use of radioactive proline.

Nitrous acid reaction

Table IV shows that conversion of the imino acids to their nitroso derivatives does not significantly affect the R_f values. Colorimetric determination of nitrous acid treated imino acids gives the same results as the imino acids themselves.

Under the excess nitrous acid conditions used, all of the amino acids are effectively deaminated. The effect of nitrous acid on a hydrolysate of gelatin is shown in Figure 4, in which the amounts of hydrolysate and isolated imino acids seen on the chromatogram are all related to 1 mgm of gelatin.

Removal of amino acid derivatives

A ninhydrin stained chromatogram of an amino acid mixture which has been nitrous acid treated will reveal only

spots of proline and hydroxyproline. However, the presence of amino acid derivatives, and also the large amount of salt introduced with the nitrous acid reagent, will interfere with separation of the imino acids. There are several ways of removing unwanted materials after the nitrous acid stage. Desalting on Dowex 50 (H^+) resin or extractions with ether are both quite effective, however the latter method is rapid and more convenient. Complete desalting was not carried out since the thin layers are not as sensitive to the presence of salt as filter paper chromatograms. However, the salt concentration was kept fairly low by the use of 70% ethanol as solvent preparatory to chromatography.

Samples of C^{14} glycine and C^{14} proline were put through the procedure and the radioactivities followed at each stage, in order to assess the efficiency of ether extractions. The results of this experiment are shown in Table V. Glycine is effectively removed by nitrous acid treatment followed by three extractions with ether, each of 5 ml, however some loss of proline also occurred. In subsequent work a single extraction with 5 ml of ether was used, which results in only 15% loss of proline, as may be seen in Table VI. Separation of proline and hydroxyproline from a nitrous acid treated hydrolysate was quite good after one extraction with ether.

Although there will be differences in the extent to which particular amino acid derivatives are extracted with ether, figures obtained by the use of labelled glycine are

a good indication of the efficiency of ether extraction since glycine accounts for approximately one-third of the total residues in collagen.

Overall recoveries

The overall recovery of proline and hydroxyproline after nitrous acid treatment, a single ether extraction and chromatography, followed by two elutions with distilled water, is shown in Table VI. After reducing the number of ether extractions and taking a slightly larger area for elution than was indicated the overall recovery was in the region of 60%, which compares favourably with other methods of separation of proline and hydroxyproline.

Multiple peak uptake curve of collagen

In 1962 Stone and Meister (56), using tritiated proline to investigate the synthesis of collagen hydroxyproline in carrageenin granuloma minced slices, observed a loss of both hydrogens from the carbon position on which hydroxylation occurs. Concurrently Ebert and Prockop (12), using similar techniques to study the synthesis of collagen in vivo, observed the loss of only one hydrogen from the carbon of proline that becomes hydroxylated. In addition, comparison of $H^3:C^{14}$ ratios of proline and hydroxyproline isolated from collagen, after administration of labelled proline, indicated that only one hydrogen was lost from proline during hydroxylation. In the skin experiment, results of which are shown in Figure 5, specific activities, expressed as d.p.m./ μ Mole hydroxyproline, are calculated on the basis of only

75% of the tritium in proline being retained in collagen hydroxyproline, as shown in Table VII.

The surprising presence of more than one peak in the uptake curve of collagen was initially established by counting purified collagen at regular time intervals after administration of C^{14} glycine and H^3 glycine. The existence of these peaks of activity, however, could conceivably have been due to the presence of a noncollagenous impurity with a different rate of uptake of glycine. The results presented here definitely exclude the possibility of the phenomenon being due to contamination, since a similar pattern of radioactivity is seen upon counting isolated protein bound hydroxyproline, practically unique to collagen. Using the thin layer chromatography method to isolate hydroxyproline, Bentley and Jackson have now demonstrated these peaks of activity in wound granulation tissue in the guinea pig. Dr. Jerome Gross, studying collagen biosynthesis in the chick embryo, has also observed this unusual variation in the uptake curve (unpublished communication).

The results of the skin experiment are in good agreement with the pattern of radioactivity in the uptake curve described by Bentley and Jackson. This demonstrates the validity of using the thin layer chromatography method of separation of proline and hydroxyproline in collagen studies which involve isotopically labelled proline. Thus, thin layer chromatography separation of proline and hydroxyproline, in studies using isotopically labelled proline, should

allow many problems of collagen and hydroxyproline biosynthesis to be investigated using a direct approach. Collagen may be studied without extensive purification, as is demonstrated by the skin experiment confirming the multiple peak uptake curve of collagen. Isolation of pure samples of both proline and hydroxyproline from purified collagens by this technique, and comparison of the rates of uptake of labelled proline at regular time intervals after administration of isotope is another useful approach in studying the early stages of collagen biosynthesis.

The double peak uptake curve of collagen was originally demonstrated by following the incorporation of labelled amino acid into collagen for 6 hours after administration of isotope. In the study presented here, in which incorporation of H^3 proline into collagen hydroxyproline was determined at intervals between 2 and 8 hours after injection, a third peak was observed at the 7 hour time point.

A possible explanation for the double peak put forward by Bentley and Jackson was that extractability of the collagen varies at certain times after synthesis. This theory was based partly on the results of Henriques, Henriques and Neuberger (19) who had studied the specific activity of the free amino acid pool at various time intervals after injection of labelled amino acid. The cell surface was envisaged as a site of alteration of extractability since Porter and Pappas (40) had observed collagen fibrils at the cell surface that could not be washed off during preparation of the

tissue for electron microscopy. However, the presence of more than two peaks of activity, in the uptake curve described here, may invalidate application of the findings of Henriques et al. to the uptake of proline into collagen hydroxyproline. This would make alterations in extractability a less likely explanation of the phenomenon.

Studies being carried out in this department, using the thin layer chromatography method, include differentiation between extracellular and intracellular uptake of H^3 proline into hydroxyproline of neutral salt soluble collagen, to determine if the peaks occur in both fractions. In addition the uptake of H^3 proline into skin collagen proline and hydroxyproline is being examined in parallel with determinations of the specific activity of proline and hydroxyproline in both the skin and plasma free amino acid pools. Comparison of the patterns of radioactivity in each of these fractions over the first few hours of collagen synthesis may provide some better explanation of the nature of these unusual peaks in the uptake curve of collagen.

In all this work the method described in this thesis has been successfully applied both to hydrolysates of proteins and more recently to proline and hydroxyproline of the free amino acid pools of both skin and plasma. It should therefore prove to be a useful and inexpensive method of separating proline and hydroxyproline in adequate amounts for useful studies on the many problems concerned with the biochemistry of collagen.

SUMMARY

A rapid method of isolating pure samples of proline and hydroxyproline from protein hydrolysates has been developed, which is suitable for multiple samples and particularly useful in studies involving radioactive isotopes. Amino acids are destroyed by treatment with nitrous acid and their reaction products removed by extraction with ether. Proline and hydroxyproline are thus isolated as n-nitroso derivatives and are separated by thin layer chromatography on cellulose. Mixtures including up to 500 μg of each imino acid are separable within 5 hours on a 250 μ thick layer. The imino acids may be eluted, determined colorimetrically and radioactivity measured by scintillation counting.

Studies using isotopically labelled imino acids are thus possible by this simple technique, even when low specific activities are involved. Application of the technique to collagen studies allows determination of the specific radioactivity of protein bound hydroxyproline to be performed on relatively crude protein extracts.

Using this technique, the incorporation of H^3 proline into skin collagen hydroxyproline has been studied between 2 and 8 hours after injection of isotope into guinea pigs. The results confirm the multiple peak uptake curve of collagen described by Bentley and Jackson in studies employing labelled glycine and also demonstrate the validity of the method. Since protein bound hydroxyproline is practically unique to collagen, the results presented here definitely

exclude the possibility that contamination of collagen can be the explanation of the peaks of activity found in the uptake curve.

APPENDIX

Hydroxyproline Measurement by the Method of Woessner (58)

Standards: Prepare a stock solution by dissolving 25 mgm of vacuum dried L-hydroxyproline in 250 ml of 0.001 N HCl. Working standards are prepared daily by diluting the stock solution with distilled water to obtain concentrations of 0.5 - 2.5 μgm/ml.

Buffer:
50 gm of citric acid monohydrate
12 ml of glacial acetic acid
120 gm sodium acetate trihydrate
34 gm sodium hydroxide

Make to 1 liter with distilled water and adjust to pH 6.0.

Chloramine T (sodium p-toluene sulfonchloramide)
1.41 gm chloramine T
20 ml of distilled water
30 ml of methyl cellosolve
50 ml of buffer

Perchloric acid: 3.15 M solution in distilled water.

p-Dimethyl aminobenzaldehyde (PDAB): 20% (w/v) solution in methyl cellosolve, dissolved by warming to 60° C.

Method: Protein hydrolysates are neutralized and diluted, to the range of 0.5 - 2.5 μg/ml. Two ml samples of the unknowns and standards are placed in 16 x 150 mm test tubes. Oxidation of hydroxyproline is initiated by adding 1 ml of chloramine T to each tube. The tube contents are mixed and allowed to stand at room temperature for 20 minutes. Chloramine T is then destroyed by adding 1 ml of perchloric acid to each tube, mixing and standing for 5 minutes. Finally 1 ml of PDAB solution is added, and the mixture well shaken. Tubes are placed in a 60° C water bath for 20 minutes,

cooled in an ice bath. The color is stable for at least 1 hour. Optical densities are read at 557 mμ and hydroxyproline determined from the standard curve.

Proline Determination by the Method of Troll and Lindsley (57)

Standards: Solutions of proline containing 1 - 5 μgm/ml.

Ninhydrin reagent: 1.25 gm of ninhydrin dissolved by heating to 30° C in 30 ml glacial acetic acid and 20 ml 6 M H_3PO_4.

Citrate buffer pH 4.0: Permutit removes interfering substances such as lysine and ornithine quantitatively from solution at this pH.

Method: Protein hydrolysates are neutralized and diluted to the working range with citrate buffer. Ten ml of the unknowns and standards are shaken with one-tenth of their weight of "permutit according to Folin" resin (decalso) for 5 minutes. To 5 ml of this solution is added 5 ml of glacial acetic acid and 5 ml of ninhydrin reagent. The tubes are heated in a boiling water bath for 1 hour in plastic screw capped tubes. The solutions are then cooled in an ice bath and extracted with 10 ml of benzene by shaking vigorously for 5 minutes. The phases are separated, by filtration if necessary, and the optical density of the benzene phase read in a colorimeter at 515 mμ.

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