THE INVESTIGATION OF AMINO ACID REACTIONS BY METHODS OF NON-AQUEOUS TITRIMETRY

II. DIFFERENTIAL ACETYLATION OF HYDROXY GROUPS, AND A METHOD FOR THE PREPARATION OF THE O-ACETYL DERIVATIVES OF HYDROXYAMINO ACIDS*

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The present paper is intended as a contribution to the specific chemistry of the naturally occurring hydroxyamino acids. Certain established differences in the chemistry of the acyl derivatives of amino and hydroxy groups have so far not been utilized in the study of hydroxyamino acids, and in particular no simple method for the preparation of their O-acetyl derivatives has been developed. The long known catalytic effect of strong acids in the reaction of hydroxy groups with acetic anhydride has been the object of a quantitative study in aqueous medium (Conant and Bramann (4)). On the other hand, it also has long been recognized (Pinnow (11)) that the acetylation of primary amines by acetic anhydride is greatly inhibited when they are present as salts of strong acids.1

The preceding paper (Kolb and Toennies (6)) contains evidence indicating that the acetylation of amino acids by acetic anhydride in aqueous medium is greatly repressed by salt formation with perchloric acid, and an earlier study (Toennies and Elliott (20)) dealt with the accelerating effect in the same medium of perchloric acid on the acetylation of water by acetic anhydride. The present work shows that the catalyzed reaction of the —OH group of amino acids is very similar to that of water. The analytical determination of that reaction and its differentiation from the acetylation of amino groups is accomplished as follows: If an acetic solution of an

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1 Whenever acidity has been found beneficial in the acetylation of amines (Vorländer and Mumme (23), Reverdin and Crépieux (12), Smith and Orton (13)), the latter have been compounds of very weak basicity in which the extent of salt formation may be assumed to be negligible. That in those cases the accelerating effect of strong acids should prevail is logical if the latter involves an activation of the acetic anhydride rather than of the substrate to be acetylated, a view which is borne out by available evidence on the formation of active compounds between acetic anhydride and strong acids (Stillich (15), Murray and Kenyon (9)).
amino acid, acidified by perchloric acid and containing acetic anhydride, is added to an amount of anthranilic acid (or other suitable amine) which is more than equimolar to the sum of the free perchloric acid and the available acetic anhydride present, then the free amino groups will react with the available acetic anhydride forming N-acetyl derivatives. To the extent that the latter are devoid of basic properties titration with perchloric acid, after completion of the reaction between acetic anhydride and amino groups, will determine the remaining amino groups, and, if their initial amount (including those of anthranilic acid) is known, the amount which has reacted with, i.e. which is equimolar with, the available acetic anhydride. The difference between this and the initially used acetic anhydride will be a measure of the acetic anhydride consumed by reaction with —OH groups while the solution was in the acid state. However, a kinetic study of the acid-catalyzed reaction of hydroxy groups with acetic anhydride will be possible only if in the basic state the reaction of —OH with acetic anhydride is negligibly slow compared with that of —NH₂. This was found to be the case. On the other hand, the acetylation of —NH₂ groups while the solution is in the acid state was not found to be entirely negligible. The extent of this reaction is determined by a separate titration with aceticous sodium acetate, which will reveal conversion of basic —NH₂ groups to non-basic acetamino groups, but the determination of the O-acetylation will not be affected by it. Problems of analytical detail are discussed in the experimental part.

The finding that the speed of O-acetylation increases with increasing concentration of perchloric acid while the speed of N-acetylation decreases showed the way for a practical method for the preparation of O-acetyl derivatives of hydroxyamino acids. Acetylation with acetic anhydride in the presence of an excess of perchloric acid and decomposition of the remaining acetic anhydride by water is followed by neutralization with amylnamine, of which the perchlorate is soluble in practically all organic solvents. The O-acetyl derivatives of serine, threonine, tyrosine, and hydroxyproline are in their isoelectric form only moderately soluble in acetic acid, and by lowering their solubility through addition of ether or similar liquids they can be precipitated in yields of 80 to 90 per cent. By comparison the solubility of the N-acetylamino acids in the organic media used is high; so that any that may be present as by-products are eliminated in the precipitation process.

Heretofore O-acetylamino acids have apparently not been prepared. Suggestions as to their probable properties and as to possible alternative modes for their formation could be gleaned from some available data per-

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2 To our knowledge this scheme for the determination of acetic anhydride was first utilized by Kilpi (5).
taining to the analogous O-benzoyl compounds. Sørensen and Andersen (14), later confirmed by Synge (16), showed that in O-, N-diacetyl derivatives alkaline hydrolysis causes easy cleavage of the O-acyl group without affecting the N-acyl bond. That conversely under conditions of acid hydrolysis the N-acyl linkage is split more easily than the O-acyl linkage was shown by Synge (17). The opposite relation to acidity of the two types of acyl derivatives extends even further. Just as N-acylation is inhibited by acid conditions, O-acylation is inhibited by alkaline conditions. This is indicated in the study of Sørensen and Andersen (14) who found that in treating α-amino-δ-hydroxyvaleric acid with benzoyl chloride in a strongly (0.5 N) alkaline medium only the —NH₂ groups are benzoylated, whereas the —OH group also reacts when the procedure is carried out in a nearly neutral medium. Similarly Bergmann and Zervas (3) and du Vigneaud and Meyer (22) have shown that treatment of tyrosine with acetic anhydride leads to the formation of the N-acetyl derivative when the aqueous medium is strongly alkaline, while under less alkaline conditions the O-, N-diacetyl compound results. Finally, reference must be made to the studies of Bergmann et al. (1, 2) on rearrangements in acyl derivatives of hydroxyamino acids; again it emerges that acidity favors O-acylation, while alkalinity favors N-acylation.

Some of the relations outlined have been utilized for the preparation of amino acid O-acyl derivatives by Bergmann et al. (1, 2) and by Synge (17). The former obtained the O-benzoyl derivatives of γ-amino-δ-hydroxybutyric acid and serine from the corresponding N-benzoyl compounds by anhydration to the 2-phenyloxazoline ring and its reopening under acid conditions. The latter obtained O-benzoylhydroxyproline and O-benzoylserine in small yields by acid hydrolysis of the N-acetyl-O-benzoyl derivatives. Our approach to the corresponding O-acetyl derivatives is more direct as well as productive of better yields than these methods. Moreover, its applicability is not limited to compounds in which the hydroxy and the amino groups are attached to adjoining carbon atoms, as is the oxazoline method. Suitable modifications of our procedure, involving the use of benzoic or other anhydrides and the corresponding or inert solvents, may permit its extension to the preparation of other O-acyl derivatives. The preparation of O-, N-diacetyl derivatives, from the O-acetyl derivatives by the technique outlined in the preceding paper (Kolb and Toennies (6)), should offer no difficulties.

EXPERIMENTAL

Analytical Reagents—The acetic anhydride used in the present work was analyzed by several methods, with the following results. Our customary methylate method (Toennies and Elliott (20)) gave 9.29 mm of acetic
anhydride per gm., the iodometric dichloroaniline method of Orton and Bradfield (10) gave 9.30 mM, and the anthranilic acid method (detailed below) gave 9.32 mM; the average value is 9.30 ± 0.01.

A standard acetous perchloric acid solution3 was prepared by adding to a weighed amount of concentrated aqueous perchloric acid, dissolved in acetic acid (u.s.p.), an amount of acetic anhydride equimolar to the water accompanying the perchloric acid.4

When anthranilic acid as purchased was not of satisfactory purity (white crystals, titration value against acetous perchloric acid at least 99.5 per cent of the theoretical), it was purified by several recrystallizations from water and from alcohol. Fresh acetous solutions of anthranilic acid show a characteristic bluish fluorescence, and on standing develop a yellow color. This discoloration process, very rapid in direct sunlight, is practically eliminated in the dark. Besides, a very slow spontaneous acetylation occurs, but its extent is negligible for at least 20 hours in an approximately 0.5 M solution in anhydrous acetic acid.

Determination of Acetic Anhydride with Anthranilic Acid—In contrast to the behavior of acetyl- and formylalanine (Kolb and Toennies (6)) the basic effect of acetylanthranilic acid in the perchloric acid titration is

3 In defining normalities of acetous solutions in the present work effective normalities have been used throughout unless otherwise stated; i.e., (a) the normality has been corrected for the temperature of the titration by using the expansion coefficient 0.107 per cent per degree, and (b) instead of deducting the experimental solvent blank correction (cf. Toennies and Callan (18)) for the volume titrated, this correction has been incorporated in the normality value. For instance, in reference to the perchloric acid solution mentioned it was found that 10 cc. of the acetic acid used in its preparation have a blank value of 0.07 cc. of 0.1 N HClO4. Only a single end-point, viz. the point "at which emerald-green has just turned yellow-green," was used in the present work. The selection of a suitable end-point shade is a matter of the individual preference of the operator. In the standardization of the perchloric acid solution against glycine (Toennies and Callan (18)), instead of making pro rata deductions of 0.07 cc. per 10 cc. consumed, the normality was calculated without making deductions. A value (at 25°) of 0.0996 N (±0.1 per cent) was obtained, and this value was used in practice as the titrating normality. However, by making the proper blank correction a value of 0.1003 N would result for the true normality, and this value compares well with the value of 0.1004 N obtained by calculation from the amount of aqueous perchloric acid used.

4 In making up this solution the following technique, convenient for obtaining desired amounts of concentrated reagent solutions, was used. A 25 cc. glass-stoppered volumetric flask, freshly cleaned with bichromate-sulfuric acid and dried, was filled with some perchloric acid, thoroughly rinsed, emptied, drained for 60 seconds, and weighed. The desired amount of perchloric acid was then weighed into the flask, and by emptying it under the same conditions of draining as before the weighed amount is obtained within close limits.
nearly negligible: 0.6 mM of acetylanthranilic acid titrated in the presence of 10, 20, or 30 cc. of acetic acid consumed 0.07, 0.13, and 0.19 cc. of 0.1 N HCIO₄, while the corresponding solvent blanks were 0.06, 0.12, and 0.18 cc.

In order to establish the rate of reaction between acetic anhydride and anthranilic acid under the conditions encountered in the intended kinetic experiments, acetic acid solutions of the two compounds were combined in such amounts as to produce a solution of 0.093 M acetic anhydride and 0.139 M anthranilic acid. The mixture was kept in a bath at 25.30° ±0.05°, and, at intervals, 5 cc. portions were withdrawn and titrated with acetic perchloric acid, with the following results.

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>1400</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCIO₄, m.eq.</td>
<td>0.427</td>
<td>0.309</td>
<td>0.231</td>
<td>0.235</td>
<td>0.233</td>
<td>0.232</td>
<td>0.232</td>
</tr>
</tbody>
</table>

These results are in harmony with a bimolecular velocity constant (moles per liter per minute) of 0.156 (±4 per cent), and it may be concluded from them that as long as the temperature and the ratio as well as the absolute values of the concentrations are not lower than those used here a period of 3 hours is amply sufficient for complete reaction.

In a practical method for the determination of acetic anhydride account must be taken of the water ordinarily present in the acetic acid used as the medium. Its concentration is, according to our experience, approximately 0.1 M. Its effect was examined as follows: Into seven 50 cc. glass-stoppered Erlenmeyer flasks, identical 4 cc. portions of an 0.5 M anthranilic acid solution were pipetted. To two of these, 10 cc. portions of a freshly prepared approximately 0.13 M solution of acetic anhydride (exactly weighed) were added. This occurred approximately 5 minutes after the concentrated acetic anhydride had been diluted with acetic acid. 10 minutes later another two 10 cc. portions of the same solution were added to anthranilic acid flasks. All flasks (three of them as controls) were titrated after 3 hours standing. The experiment was repeated after the water content of the acetic acid used in dissolving the acetic anhydride was increased by 0.1 mole per liter. In other experiments some organic hydroxy compounds in 0.1 M concentration were added, instead of water, to the acetic acid. The compounds used were ethanol, menthol, resorcinol.

5 Acetylanthranilic acid was simply obtained by dissolving 100 mM of anthranilic acid in 150 cc. of acetic acid and adding 100 mM of acetic anhydride. The acetyl derivative crystallized; it was filtered after several hours and dried in the air in a warm place. Yield about 60 per cent. Equivalent weight (NaOH, phenolphthalein) found 177.7, calculated 179.1.
and hydroquinone. In all cases (addition of water and of hydroxy compounds, time of interaction with acetic anhydride previous to combination with anthranilic acid solution either 5 or 15 minutes) the resulting value for acetic anhydride was within ±0.2 per cent of that obtained with unadulterated acetic acid. One may conclude, therefore, that under the experimental conditions organic hydroxy groups are not likely to interfere with the determination of acetic anhydride, and that, likewise, moderate amounts of water can be disregarded.

On the other hand, in the kinetic experiments described below the solution in which the amount of free acetic anhydride is to be determined contains free perchloric acid which in the concentrations present (0.02 M) renders the interaction of water with acetic anhydride extremely rapid (cf. Toennies and Elliott (19)). Rather than to determine whether the acid can be a cause of errors by producing a certain amount of reaction of anhydride with the water present in the anthranilic acid solution, during the process of adding the acidified anhydride solution to the latter, it was considered simpler to employ dehydrated acetic acid for the anthranilic acid solutions.

Preparation and Analysis of Dehydrated Acetic Acid—To 10 liters of acetic acid 200 cc. of 10.04 M acetic anhydride and 100 cc. of 0.100 N aceticous perchloric acid were added. After 2 days the resulting solution was analyzed for free acetic anhydride in the manner described, on 10 cc. portions, with 4 cc. portions of 0.5 M aceticous anthranilic acid. The difference in titration between tests and anthranilic acid blanks (three of each) was 9.42 ± 0.02 cc. of 0.100 N HClO. From this must be deducted 0.10 cc. for the perchloric acid present (0.001 M) in the solution that is being analyzed, so that an acetic anhydride concentration of 0.0932 mole per liter results for the dehydrated solution, or a total amount, assuming additive volumes, of 960 mm. Since 2008 mm of acetic anhydride were added, 1048 mm of water have reacted; i.e., the original water content was 0.104 mole per liter. The analysis of the dehydrated solution was repeated, after 2 months standing, with essentially the same results.

The blank titration value of the ordinary acetic acid is about 0.15 cc. of 0.1 N HClO per 25 cc.; that of the dehydrated solution corresponds to only 0.05 cc. per 25 cc., since back titration of the solution, which according to the perchloric acid present should take 0.25 cc. of 0.1 N aceticous sodium acetate, requires only 0.20 cc.

Preparation and Analysis of Anhydrous Perchloric Acid Solutions—In measurements of the acid-catalyzed acetylation of hydroxy groups water is, of course, a source of error. For this reason perchloric acid solutions as nearly anhydrous as possible were prepared for use in the reaction mixtures to be investigated. Three different concentrations of perchloric acid, 0.10 M, 0.125 M, and 0.50 M, were prepared in this connection.
To 1000 cc. of the dehydrated acetic acid (0.001 M HClO₄, 0.0932 M (CH₃CO)₂O) 14.57 gm. of HClO₄ (68.42 per cent) and 16.53 gm. of (CH₃CO)₂O (95.1 per cent) were added. According to calculation the resulting solution should be 0.0978 M in HClO₄ and should have a residual water content of 0.009 mole per liter. Standardization by titration of dry samples of anthranilic acid and against a standardized sodium acetate solution gave the value of 0.0976 ± 0.002 n for HClO₄, and instead of the estimated residual water content a slight excess of acetic anhydride was found, by using the following analytical procedure.

A 6 cc. portion of a 0.5 M anthranilic acid solution was titrated with the perchloric acid solution to be analyzed, 30.80 cc. being required. By running this amount into another flask, adding a 6 cc. portion of anthranilic acid to it, and completing the titration, the total HClO₄ required was (two experiments) 30.87, 30.90 cc. The higher value is presumably due to the fact that in the first experiment (direct titration) some of the anthranilic acid (base) is “neutralized” by being acetylated by acetic anhydride present in the HClO₄ solution. If the two solutions are combined in the reverse order, the prevailing acid reaction will prevent the acetylation. Now three additional 6 cc. portions of the anthranilic acid solution were pipetted out and 10.00 cc. of perchloric acid were added to each. After 4 hours one was titrated, resulting in a total consumption of 30.52 cc. of HClO₄ solution. When this amount was added rapidly to the other two flasks and then the titration completed, 30.52 and 30.54 cc. were used. If the difference between the total anthranilic acid titrated above (30.89 ± 0.02 cc. of HClO₄) and that titrated after the solution had been in contact with 10 cc. of the HClO₄ solution for 4 hours is assumed to be the result of acetylation by acetic anhydride present in the 10 cc., an acetic anhydride content of the perchloric acid solution of 0.0035 mole per liter is indicated.

By similar procedures 1 liter of a 0.1252 N HClO₄ solution was prepared and analyzed. In this case the balance between acetic anhydride and water should, according to calculation, have resulted in a residual water content of 0.0005 mole per liter; analysis, however, indicated the presence of 0.0034 M acetic anhydride.

Finally 1 liter of a 0.5 M anhydrous HClO₄ solution was prepared, likewise by first dissolving the concentrated aqueous perchloric acid in the dehydrated acetic acid and then adding the requisite amount of acetic anhydride. As in this case the heat of reaction was considerable and since the resulting solution is notably more viscous than the less concentrated

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6 Other, unpublished, experiments support the suggestion conveyed by these data that the reaction (CH₃CO)₂O + H₂O → 2CH₃COOH may not be entirely irreversible.
ones, it was deemed neither safe nor expedient to operate with anhydrous
acetic perchloric acid solutions more concentrated than 0.5 M.

Inhibition of N-Acetylation by Acidity—In the preceding paper (Kolb
and Toennies (6)) an experiment has been presented which shows that the
N-acetylation of an amino acid (alanine) is extremely slow in the presence
of an excess of perchloric acid. In the present work additional data on
this point, related to the conditions employed in the study of the hydroxy
acetylations, have been obtained. Equal amounts (216.8 mg.) of dl-
alanine were dissolved (a) in 25.02 cc. of an acetic perchloric acid solu-
tion (0.0973 N HClO₄, 0.0032 M (CH₃CO)₂O), i.e. an equivalent amount;
(b) in 25 cc. of a solution obtained by diluting 0.5 cc. of an anhydrous

<table>
<thead>
<tr>
<th>Time</th>
<th>0.000 M excess HClO₄</th>
<th>0.004 M excess HClO₄</th>
<th>0.015 M excess HClO₄</th>
<th>0.036 M excess HClO₄</th>
<th>0.072 M excess HClO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>CC. per cent.</td>
<td>CC. per cent.</td>
<td>CC. per cent.</td>
<td>CC. per cent.</td>
<td>CC. per cent.</td>
</tr>
<tr>
<td>0.15</td>
<td>0.20</td>
<td>0.36</td>
<td>0.79</td>
<td>1.90</td>
<td>3.94</td>
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<td>0.5</td>
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<td>0.26</td>
<td>0.53</td>
<td>0.70</td>
<td>0.70</td>
</tr>
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<td>1.0</td>
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<td>0.25</td>
<td>0.80</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>11</td>
<td>0.26</td>
<td>0.26</td>
<td>0.82</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>22</td>
<td>0.32</td>
<td>0.34</td>
<td>0.82</td>
<td>0.70</td>
<td>0.70</td>
</tr>
</tbody>
</table>

0.5 M HClO₄ solution to 50 cc. with the solution mentioned under (a); (c) similarly except that the amount of 0.5 M HClO₄ used was 2 cc.; (d) similarly except with 5 cc. of 0.5 M HClO₄; and (e) similarly except with 10 cc. of 0.5 M HClO₄. Each solution was combined with 2.5 cc. of a 1.42 M solution of acetic anhydride, and at intervals 18.0 per cent aliquots (5 cc., determined by weight) were withdrawn and titrated with a 0.1006 N acetic sodium acetate solution. The experiments were conducted at 25.4° ±0.2°. The results are summarized in Table I. It appears that the rate of N-acetylation decreases with increasing acidity, but that prac-
tically no further suppression is achieved by an excess of perchloric acid
greater than 20 per cent.

Reaction of Hydroxyamino Acids with Acetic Anhydride in Presence of
Perchloric Acid—The compounds used for the kinetic experiments were
dried, pulverized, passed through a 100 mesh sieve, and dried again, for 1 hour periods, at 100° until of constant weight. Samples were then analyzed by perchloric acid titration, with the following results: hydroxyproline, equivalent weight found 131.1 (theoretical 131.1), serine 105.3 (theoretical 105.1), threonine 119.0 (theoretical 119.1), tyrosine 180.9 (theoretical 181.1).

The reaction of the four available hydroxyamino acids with acetic anhydride in acetic acid was studied under two conditions of acidity, Series A in the presence of an equimolar amount of HClO₄, and Series B in the presence of an excess of approximately 28 per cent, while in both cases the amino acid concentration was about 0.09 M, and about 1.4 molecules of acetic anhydride were available for each molecule of amino acid. Furthermore, in one case (Series C, tyrosine) the course of the reaction was followed at substantially higher concentrations, viz. approximately 0.4 M amino acid, in the presence of 1.2 molecules of perchloric acid and 1.4 molecules of acetic anhydride per molecule of amino acid, in order to establish the absence of complications when the concentrations are multiplied. This experiment was carried out primarily with a view toward the isolation of the expected O-acetyl derivatives.

The procedures were as follows: In Series A, anhydrous approximately 1.4 M acetic anhydride solution was prepared by diluting 15 cc. of 95.1 per cent acetic anhydride, together with 10 cc. of 0.1 N aceticus HClO₄, to 100 cc. with ordinary acetic acid. After 24 hours were allowed for the acid-catalyzed acetylation of the available water, the acetic anhydride content of the solution was determined by the anthranilic acid (4 cc. of 0.5 M) method on weighed 0.9 cc. portions. Then exactly 5.00 mm of the respective amino acid were weighed out into a 100 cc. glass-stoppered flask, and a volume of an anhydrous, approximately 0.1 N (acidity value and that of the small excess of acetic anhydride present accurately determined) HClO₄ solution was added which corresponds to 4.95 milliequivalents. After the amino acid had dissolved, the flask was placed in the bath at 25.4° ±0.1° and, after at least 10 minutes were allowed for temperature adjustment, 5 cc. of the above acetic anhydride solution were added, with thorough agitation. Since the acetic anhydride solution is 0.01 N in HClO₄, the total perchloric acid is now 5.00 mm; i.e., equivalent to the amino acid. At selected intervals, counting from the time of addition of acetic anhydride, 5 cc. portions of the reaction mixture were withdrawn and immediately titrated back with 0.1 N sodium acetate solution. Other 5 cc. portions were directly pipetted into 2 cc. portions of a 0.5 M anthranilic acid solution and, after 3 hours, titrated with aceticus HClO₄, together with anthranilic acid blanks. The amount of perchloric acid solution used in the reaction mixture had been weighed; by determining the weight of 5
cc. portions of the acetic anhydride solution used and of some of the 5 cc. portions withdrawn from the reaction mixture for analysis, all necessary data were obtained for calculating the composition of the reaction mixture and the weight fraction of the analytical portions, so that the results could be calculated in terms of the total reaction mixture without the complicating necessity of making the reaction mixtures to a definite volume and other difficulties. In the reactions of Series B the procedure was similar except that the perchloric acid used in the reaction mixture was about 0.125 N. In the single experiment which was carried out at a substantially higher concentration, Series C, 4.96 mM of tyrosine were dissolved in 12 cc. of an anhydrous 0.503 M perchloric acid solution. After the mixture was weighed and had reached the temperature of the bath, concentrated acetic anhydride corresponding to 7.20 mM was added, and 1 cc. portions of the reaction mixture were used for the determinations.

The results of these experiments are presented in Table II. It appears that the rate of N-acetylation, both in the absence of free perchloric acid and in its presence, is lowest in the case of tyrosine and highest in the case of hydroxyproline. Furthermore, the velocity of this reaction is, in confirmation of what had already been demonstrated for alanine (Table I), in all cases decreased by the presence of free perchloric acid. The velocity of O-acetylation, on the other hand, is always increased by the addition of acid. Among the different compounds the reaction of the hydroxy groups seems likewise to be slowest in the case of tyrosine and fastest in the case of hydroxyproline, but the relative position of the other two representatives, serine and threonine, may not be the same here as in the reactivity of the amino groups. In appraising the order in which the reactivity of the four compounds appears in experimental Series A, one must consider the possibility that the relative position may in part be the result of small variations, beyond the experimental precision, in the acid-base ratio, since the balance in this series is one of "neutrality" (NH$_2$ equal to HClO$_4$); i.e., possibly a range highly sensitive to small variations. However, the fact that the relative order of the four compounds is the same in the "neutral" series as in that containing an excess of acid speaks against the importance of this source of error.

The small deviations from the theoretical value of 100 per cent which are in evidence in the O-acetylation data of Table II are thought to be the combined result of imperfect purity of the compounds used and of analytical errors. The latter are especially prominent in the more concentrated system of Series C where increased viscosity of the solution and small size of samples are distinct sources of error.

Preparation of Acetoxyamino Acids—The general procedure developed for purposes of isolation is as follows: By appropriate dilution of the con-
centrated aqueous perchloric acid with acetic acid an acetic solution 0.60 M in HClO₄ and about 1.7 M in H₂O is prepared. 100 cc. of this solution are added to 50 mM of the pulverized hydroxyamino acid. After solu-

### Table II

**Rates of Acetylation of Amino and Hydroxy Groups of Hydroxyamino Acids**

The experimental conditions described in the text were used. The values are given in per cent.

<table>
<thead>
<tr>
<th>Time*</th>
<th>Tyrosine</th>
<th>Serine</th>
<th>Threonine</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-NH₂</td>
<td>-OH</td>
<td>-NH₂</td>
<td>-OH</td>
</tr>
<tr>
<td>Series A. 0.088 M amino acid, 0.088 M perchloric acid, 0.128 M acetic anhydride</td>
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</tr>
<tr>
<td>hrs.</td>
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<tr>
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<td>98.8</td>
<td>16.2</td>
<td>98.7</td>
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<tr>
<td>Series B. 0.088 M amino acid, 0.113 M perchloric acid, 0.123 M acetic anhydride</td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>0.6</td>
<td>102.2</td>
<td>0.8</td>
<td>102.4</td>
</tr>
<tr>
<td>14.5</td>
<td>0.6</td>
<td>102.2</td>
<td>0.8</td>
<td>102.4</td>
</tr>
<tr>
<td>Series C. 0.368 M amino acid, 0.447 M perchloric acid, 0.530 M acetic anhydride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>0.0</td>
<td>103</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.2</td>
<td>103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>103</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>0.2</td>
<td>103</td>
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<td></td>
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<tr>
<td>1.5</td>
<td>103</td>
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<td></td>
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<tr>
<td>1.75</td>
<td>0.2</td>
<td>103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>103</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In Series A and B the determination of —NH₂ acetylation (titration with sodium acetate) was performed approximately 5 minutes after the stated times.
conditions the heat of acetylation is considerable, it is advisable to run
the concentrated acetic anhydride slowly into the solution, which is cooled
by gentle swirling in an ice bath. The combined solution is kept in a glass-
stopped flask for about 1 hour at room temperature, in order to insure
completion of the O-acetylation. Thereupon 2 cc. of water (110 mM)
are added which under the prevailing conditions of acid catalysis will
eliminate all remaining acetic anhydride. If the latter were not thus
removed, it would react with amino groups when the solution is made
basic in the next step, and thus could interfere with yield and purity of the
reaction product. After 1 hour is allowed for the hydration of the residual
acetic anhydride, 80 mM of commercial amylamine (Sharples Solvents
Corporation; approximately 8.0 m) are added to the solution with cooling.
In some cases the reaction product will begin to precipitate on standing;
it is obtained promptly and in good yield by the addition of a suitable
precipitating liquid, such as alcohol, acetone, ether, chloroform, etc. The
exact choice for each compound under consideration is best determined
by experiment, and the solvent combinations stated below have been thus
found. After at least one night in the refrigerator the precipitate is
filtered and washed with ether until the acidity of the washings does not
decrease further. The product is cautiously (some O-acetyl derivatives
show signs of decomposition at 100°) dried to constant weight and analyzed.
Data about the individual compounds are summarized in Table III.
The evidence for the identity of the isolated products consists of (a) the
data of Table II which show that the amino acids used consume quan-
titatively 1 molecule of acetic anhydride without involvement of the
amino group, (b) the equivalent weights obtained by perchloric acid and
methylate titrations which are in harmony with an increase of the molec-
ular weight of the parent compound by the value corresponding to CH₃CO,
and which remain essentially unchanged after a fractionating recrystalliza-
tion, (c) the essentially identical equivalent weights of the compounds as
bases (by HClO₄) and as acids (by methylate), which are indicative of the
amphoteric nature of a monoaminomonocarboxylic acid, and (d) a polarimi-
etric analysis in the case of the tyrosine and hydroxyproline derivatives
(see below).

The observed deviations in the acid values do not seem to be due entirely
to lack of purity but may be related to the basic properties of the com-
ounds (cf. Toennies and Callan (18)). All perchloric acid titrations
could be carried out without the use of formic acid, although the recryst-
tallized specimens, because of their more coarsely crystalline nature, re-
quired considerable intermittent heating in order to complete the titrations.
As stated in Table III, the tyrosine derivative showed abnormal behavior

7 It is convenient to determine this by titrating portions of the ether washings
with sodium methylate and thymol blue indicator (Lavine and Toennies (7)).
**Table III**

**Preparation of Acetoxyamino Acids**

For other details, see the text.

<table>
<thead>
<tr>
<th>Starting material</th>
<th>O-Acetyl derivative, isolation</th>
<th>Recrystallization of derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equivalent weight found*</td>
<td>Decomposition point</td>
</tr>
<tr>
<td></td>
<td>(Found CH₃COOH)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solvent combination</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yield</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Theoretical mol. wt.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equivalent weight found* by</td>
<td></td>
</tr>
<tr>
<td></td>
<td>titration with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solvent (90% in methanol)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Equivalent weight found* by</td>
<td></td>
</tr>
<tr>
<td></td>
<td>titration with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decomposition point</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>°C.</td>
<td></td>
</tr>
<tr>
<td>l-Hydroxyproline</td>
<td>130.7</td>
<td></td>
</tr>
<tr>
<td>(131.1)</td>
<td>200 cc. butyl ether + 2000 cc.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ethyl ether†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 cc. butyl ether + 2000 cc.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ethyl ether</td>
<td></td>
</tr>
<tr>
<td>dl-Serine</td>
<td>106.3</td>
<td></td>
</tr>
<tr>
<td>(105.1)</td>
<td>1200 cc. ethyl ether</td>
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<tr>
<td>dl-Threonine</td>
<td>117.6</td>
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<tr>
<td>(119.1)</td>
<td>50 cc. butyl ether + 1000 cc.</td>
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</tr>
<tr>
<td>l-Tyrosine</td>
<td>184.7</td>
<td></td>
</tr>
<tr>
<td>(181.1)</td>
<td>250 cc. acetone + 750 cc. ethyl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ether</td>
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<tr>
<td></td>
<td>83</td>
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<td></td>
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<tr>
<td></td>
<td>149.5</td>
<td></td>
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<tr>
<td></td>
<td>147.3</td>
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</tr>
<tr>
<td></td>
<td>149.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>143–144§</td>
<td></td>
</tr>
<tr>
<td></td>
<td>164.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>162.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>146–149§</td>
<td></td>
</tr>
<tr>
<td></td>
<td>224.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>213–214</td>
<td></td>
</tr>
</tbody>
</table>

* The figures given are the averages of determinations carried out at least in duplicate.

† 0.1 N CH₃ONa, standardized against benzoic acid; indicator, thymol blue; end-point, from yellow to blue; blank correction to be deducted obtained by titrating to the same color after addition to the titrated solution of methanol and indicator in amounts equal to those already present.

† The precipitate was oily at first, but after 5 days in the cold, with repeated agitation, all had solidified. In another experiment on a smaller scale (5 mM) a well crystallized product was obtained at once by the use of 2½ volumes of methyl cellosolve (CH₃OCH₂CH₂OH) and 20 volumes of ether.

§ With gas evolution.

|| No definite end-point could be obtained; apparently the acetyl group is slowly split off by the methylate.

in the methylate titration. Titration of the new compounds by the formol method was attempted, but no definite end-points could be obtained because of hydrolysis of the compounds on addition of alkali.

Comparison of the decomposition points (last column, Table III) with
those listed in the literature for the four parent compounds reveals a surprisingly consistent drop of close to 100° as a result of the O-acetylation.

A polarimetric analysis was carried out on the two compounds derived from optically active starting material. 2 dm. tubes were used.

O-Acetyl-\(l\)-tyrosine 0.276 M, in 1.10 M HCl, \(\alpha_D^{25} = -1.13°\), \(\alpha_{Hg}^{25} = -1.31°\) or \([M]_{D}^{25} = -23.7°\); after 20 hours \(\alpha_D^{25} = -0.87°\), \(\alpha_{Hg}^{25} = -1.00°\) or \([M]_{D}^{25} = -18.1°\).

\(l\)-Tyrosine 0.276 M, in 1.10 M HCl, \(\alpha_D^{25} = -0.91°\), \(\alpha_{Hg}^{25} = -1.03°\) or \([M]_{D}^{25} = -18.7°\); cf. \([M]_{D}^{25} = -18.4°\) (Winnek and Schmidt (24)).

O-Acetyl-\(l\)-tyrosine 0.276 M, in 1.10 M NaOH, \(\alpha_D^{25} = -1.22°\), \(\alpha_{Hg}^{25} = -1.38°\) or \([M]_{D}^{25} = -25.0°\); after 20 hours \(\alpha_D^{25} = -1.19°\), \(\alpha_{Hg}^{25} = -1.37°\).

\(l\)-Tyrosine 0.276 M, CH\(_3\)COONa 0.276 M, in 0.82 M NaOH, \(\alpha_D^{25} = -1.22°\), \(\alpha_{Hg}^{25} = -1.41°\) or \([M]_{D}^{25} = -25.5°\); after 20 hours \(\alpha_D^{25} = -1.24°\), \(\alpha_{Hg}^{25} = -1.45°\).

O-Acetyl-\(l\)-hydroxyproline 0.050 M, in 0.50 M HCl, \(\alpha_D^{25} = -0.46°\), \(\alpha_{Hg}^{25} = -0.55°\) or \([M]_{D}^{25} = -46°\); after 20 hours \(\alpha_D^{25} = -0.50°\), \(\alpha_{Hg}^{25} = -0.62°\), after 44 hours \(-0.60°\).

\(l\)-Hydroxyproline 0.050 M, in 0.50 M HCl, \(\alpha_D^{25} = -0.60°\) or \([M]_{D}^{25} = -60°\); cf. \([M]_{D}^{25} = -63°\) (Lutz and Jirgensons (8)), \(\alpha_{Hg}^{25} = -0.76°\); after 40 hours \(\alpha_D^{25} = -0.53°\), \(\alpha_{Hg}^{25} = -0.70°\).

O-Acetyl-\(l\)-hydroxyproline 0.050 M, in 0.20 M NaOH, \(\alpha_D^{25} = -0.87°\) or \([M]_{D}^{25} = -87°\), \(\alpha_{Hg}^{25} = -1.05°\).

\(l\)-Hydroxyproline 0.050 M, CH\(_3\)COONa 0.050 M, in 0.15 M NaOH, \(\alpha_D^{25} = -0.87°\) or \([M]_{D}^{25} = -87°\), \(\alpha_{Hg}^{25} = -1.05°\).

These determinations indicate rapid hydrolysis of the \(l\)-acetyl derivatives under alkaline conditions and substantial absence of racemization in the course of preparation and hydrolysis, since in both cases the rotation of the hydrolyzed derivative was practically identical with that of the parent compound under comparable conditions. In an acid medium, on the other hand, there is evidence of a much slower hydrolytic cleavage, since the initial readings were constant for at least \(\frac{1}{2}\) hour. In the case of the tyrosine derivative a value corresponding to that of the parent compound is reached overnight, while in the case of the hydroxyproline derivative there is evidence that racemization is superimposed upon hydrolysis.

The help of J. J. Kolb in the completion of the isolation experiments is gratefully acknowledged.

**SUMMARY**

Investigation of the reactions of hydroxyamino acids with acetic anhydride, in acetic acid in the presence of perchloric acid, showed that while the acetylation of amino groups is increasingly suppressed by increasing acidity the acetylation of hydroxy groups is catalytically promoted by the perchloric acid. The consumption of acetic anhydride is determined in these experiments by a titrimetric method which is based on the fact that amino groups on acetylation tend to lose their basic properties with respect to aceticous perchloric acid. It was found that in the aceticous system, in
the presence of excess perchloric acid, the hydroxyamino acids may be quantitatively acetylated solely on the hydroxyl group. A method for the preparation of the new compounds O-acetylhydroxyproline, O-acetylseryine, O-acetylthreonine, and O-acetyltyrosine, based on the findings outlined, is presented. The method is useful for the preparation of other related compounds.

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THE INVESTIGATION OF AMINO ACID REACTIONS BY METHODS OF NON-AQUEOUS TITRIMETRY: II. DIFFERENTIAL ACETYLATION OF HYDROXY GROUPS, AND A METHOD FOR THE PREPARATION OF THE O-ACETYL DERIVATIVES OF HYDROXYAMINO ACIDS
Warwick Sakami and Gerrit Toennies


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