28. THE BEHAVIOUR OF SOME URAMIDO-ACIDS IN THE NITROUS ACID METHOD FOR THE DETERMINATION OF AMINO-NITROGEN

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IN attempting recently to identify a substance produced by the action of arginase upon carbamido-arginine one of us [Hunter, 1938] took occasion to apply to it the well-known nitrous acid method of Van Slyke for the determination of primary amino groups. The question thereupon presented itself, whether the results obtained were consistent with the supposed constitution of the product as an α -uramido- δ -amino-acid. The behaviour of a simple amino group could of course be predicted, but a search of the literature yielded surprisingly little information upon that to be expected of a uramido group. Rohde [1918] had stated that uramido-acids yield "slightly more" than 1 atom of N after 1 hr. of interaction with nitrous acid, and had proposed upon this basis a method for their determination; but her generalization rested upon no more than two examples, of which one (the uramido-acid derived from phenylalanine) had actually given 1.25 atoms and the other (from leucine) 1.12. The only other pertinent observations we could find were those of Wada [1930] upon the α -amino- δ -uramido-acid, citrulline, which had yielded in 1.5 hr. 0.63 and in 2.5 hr. (or more) exactly 1 atom of uramido- (*plus*, of course, 1 atom of amino-) N.

This paucity of information induced us to examine the behaviour of a somewhat wider range of acids, the object in view being the purely practical one of ascertaining how far the Van Slyke method might be useful in the characterization, and possibly the quantitative determination, of such compounds. Regarded from this aspect the results were only moderately encouraging, for, as will be seen, they revealed a notable diversity of behaviour. Certain limited regularities nevertheless became evident, and it seems not impossible that a wider selection of material would have enabled us to establish these regularities as general rules. In any case, since uramido-acids have frequently been encountered in biological material, it seems worth while to put on record such data as we have collected.

The survey was facilitated by gifts of material, here gratefully acknowledged, on the one hand from Dr Henry D. Dakin, on the other from Drs Edwin J. Cohn and Thomas L. McMeekin of the Department of Physical Chemistry, Harvard Medical School. From the Harvard Laboratory we received samples of the uramido-acids derived from β -alanine, *nor*leucine and ϵ -aminohexanoic acid; from Dr Dakin samples of the corresponding derivatives of *l*(+)- α -alanine, *l*(-)-phenylalanine, *dl*- α -aminobutyric acid, *dl*- α -aminoisobutyric acid, *dl*-valine, *dl*-*nor*valine, *dl*-leucine and *dl*-isoleucine. Each of these samples had been prepared by the interaction of the corresponding amino-acid with KCNO. Descriptions of all but one will be found in the papers of Lippich [1908], Dakin [1910; 1915], Dakin & Dudley [1914], West [1918], Boyd [1933] or McMeekin *et al.* [1936]. The *nor*valine derivative does not appear to have been mentioned in the

literature; the specimen supplied to us, as well as another prepared by ourselves, melted at 178–9°. Other uramido-acids included in the study were the α -uramido- δ -guanidino-, the α -uramido- δ -amino- and the α -amino- δ -uramido-derivatives of *n*-valeric acid. The first of these ("carbamido-arginine") we prepared from *l*(+)-arginine by the KCNO method [Boon & Robson, 1935], the second from the first by the action of arginase [Hunter, 1938], and the third (citrulline) from *l*(+)-arginine in the manner described by Gornall & Hunter [1939]. With the object of checking some of our first results we prepared also additional specimens of the uramido-acids from *dl*- α -alanine, *dl*- α -aminobutyric acid, *dl*-norvaline, *dl*-valine and *l*-leucine.

Observations upon the 14 uramido-acids specified were supplemented by others upon urea, upon ornithine and upon argininic acid (prepared from arginine by the method of Felix & Müller [1936]). The last two were included merely to furnish appropriate corrections for the effect of a δ -amino or δ -guanidino group.

The purity of all materials studied was checked by nitrogen determinations, the specimen in hand being, if necessary, recrystallized until the result was satisfactory. For several of the analyses we have to thank Mr Stephen M. Nagy of Dr Max Bergmann's laboratory at the Rockefeller Institute, New York.

The nitrous acid method was applied in its manometric form [Van Slyke, 1929] and always under the same conditions. The volume of uramido-acid solution analysed was 5 ml., the total volume of reacting mixture 8 ml. and the volume, at which the pressure of the nitrogen was measured, 2 ml. Throughout each determination the temperature was kept as nearly as possible at 25°. Measurements were made of the nitrogen evolved not only after 3 min. (the time estimated as sufficient for the complete decomposition of an α -amino-group), but also, in separate experiments, after 30 min. and after 2.5 hr. Sometimes an additional measurement was made after 1 hr. At the end of the 3 min. interval, timed from the instant at which half of the nitrite solution had entered the reaction chamber and terminated by 1 min. of shaking, the transfer of the evolved gases to the alkaline permanganate was made with the utmost possible celerity, so as to minimize the error due to the still rather rapidly continuing evolution of nitrogen. At the later stages the speed of this evolution had been so reduced, that the effect of small errors in the time measurement had become negligible. During the 2.5 hr. interval so much gas accumulated in the reaction chamber, that the mercury tended to rise ultimately to the top of the manometer. Before this actually occurred, the gases were passed into the permanganate, the NO absorbed and the nitrogen either left in the absorption pipette or returned to the chamber. After one such preliminary absorption the reaction could be completed, and the total liberated nitrogen measured, in the usual way.

The concentration of the solutions analysed varied from 0.033 to 0.16 % and the amount of uramido-(+amino-) N in the 5 ml. sample from 0.3 to 1.6 g. These quantities were such that the nitrogen liberated in the 2.5 hr. experiment exerted (at 2 ml. volume) a pressure of not less than 190 and (except in one instance) not more than 440 mm. of mercury. Within the limits thus defined the concentration of a given substance could, it was found, be varied with little, if any, effect upon the results (see, for instance, data in Table 1 under Nos. 9B, 13A and 13B).

Each observation made on any one solution was repeated at least once, often twice or more. In many cases the result obtained with one solution or one sample of a substance was controlled by measurement on another. The degree of consistency apparent among parallel observations was in general excellent. The most considerable discrepancy was that encountered in the comparison of two different samples of α -uramido-isohexoic acid (Nos. 11A and 11B).

Table 1. Action of nitrous acid on uramido-acids, etc.

No.	Substance	Related amino-acid	Concen- tration %	Atoms of nitrogen liberated in			
				3 min.	30 min.	1 hr.	2-5 hr.
Group I							
1	ϵ -Uramido- <i>n</i> -hexoic acid	ϵ -Amino- <i>n</i> -hexoic acid	{0.1366 0.1466}	0.28	0.58 0.58	0.61	0.66 0.66
2A	α -Uramidopropionic acid A	α -Alanine	0.0960	0.23	0.64	—	0.72
2B	α -Uramidopropionic acid B	α -Alanine	0.1112	0.24	—	—	0.68
3	δ -Uramido- α -amino- <i>n</i> -valeric acid (citulline)	Ornithine	0.0920	0.16	0.50	0.60	0.76
4	β -Uramidopropionic acid	β -Alanine	0.1294	0.28	0.72	—	0.78
Group II							
5	α -Uramido- δ -guanidino- <i>n</i> -valeric acid	Arginine	{0.0832 0.1333 0.1644}	0.28 0.27	1.01 1.02	—	1.28 1.21
6A	α -Uramido- <i>n</i> -butyric acid A	α -Amino- <i>n</i> -butyric acid	{0.0822 0.1037}	—	1.06	—	1.24 1.28
6B	α -Uramido- <i>n</i> -butyric acid B	α -Amino- <i>n</i> -butyric acid	0.1089	0.27	—	—	1.28
7	α -Uramido- <i>n</i> -hexoic acid	<i>nor</i> -Leucine	0.0724	0.19	1.08	1.20	1.30
8	α -Uramido- δ -amino- <i>n</i> -valeric acid	Ornithine	0.1008	0.30	0.92	—	1.27
9A	α -Uramido- <i>n</i> -valeric acid A	<i>nor</i> -Valine	{0.0995 0.0498}	—	1.12	—	1.31 1.35
9B	α -Uramido- <i>n</i> -valeric acid B	<i>nor</i> -Valine	0.0825	—	—	—	1.31
10	α -Uramido- β -phenylpropionic acid	Phenylalanine	0.0698	0.28	1.15	—	1.42
11A	α -Uramido- <i>iso</i> hexoic acid A	Leucine	0.0698	0.28	1.31	—	1.60
11B	α -Uramido- <i>iso</i> hexoic acid B	Leucine	0.0818	0.31	—	—	1.48
Group III							
12	α -Uramido- β -methyl- β -ethylpropionic acid	<i>iso</i> -Leucine	0.0692	0.28	1.48	—	1.98
13A	α -Uramido- <i>iso</i> valeric acid A	Valine	0.0328	0.30	1.55	1.81	2.00
13B	α -Uramido- <i>iso</i> valeric acid B	Valine	0.0603	0.32	1.57	1.77	1.99
14	α -Uramido- <i>iso</i> butyric acid	α -Amino- <i>iso</i> butyric acid	{0.1034 0.0517}	0.31	1.58	—	2.00
Miscellaneous							
15	Arginine acid	—	{0.1556 0.1870}	0.006	0.06	—	0.34
16	Ornithine monohydrochloride	—	0.0904	0.004	0.06	—	0.34
17	Urea	—	{0.1656 0.0552 0.0344}	1.86 0.12 —	(2.00) — —	— — 1.34	(2.00) — 1.76

ERRATUM

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page 195, line 2 2·5 should read “2–5” (i.e from 2 to 5)

The data secured are collected in Table 1, each figure in which is the average of 2.5 concordant observations. The order in which the uramido-compounds are arranged is that of increasing amounts of uramido-N liberated in 2.5 hr. Different preparations of any one substance are distinguished by the letters A and B. We have not thought it worth while to make note, in Table 1, of spatial configurations. For the ornithine and arginine derivatives it should be noted that the figures given represent not the total N liberated, but that part only which was estimated to have arisen from the uramido group. In the case of citrulline (No. 3) they were calculated by deducting 1 atom (representing the α -amino group) from each experimentally observed result; in the case of α -uramido- δ -guanidinovaleric acid (No. 5) by deducting at each time period the corresponding figure for argininic acid (representing nitrogen from the guanido group); in the case of α -uramido- δ -amino-valeric acid (No. 8) by deducting the nitrogen derived from the δ -amino group, this in turn being estimated by subtracting 1 atom (the α -amino-N) from the corresponding figures for ornithine (No. 16). Thus, for example, at 3 min., the actual figures were 1.16 atoms for No. 3, 0.289 for No. 5, and 1.05 for No. 8. With respect to ornithine it may be mentioned, that the time necessary for the complete liberation of its second N was found to be exactly 10 min. at 25°.

The first point evident in Table 1 is that the uramido group is decomposed by nitrous acid much less rapidly than either the α - or the ω -amino group in an amino-acid. To this extent our observations confirm those of Rohde [1918]. On the other hand it is obviously not the case that all uramido-acids liberate in 1 hr. one atom of N. The examples examined can be arranged, as shown, in three distinct, if not quite sharply delimited, groups. Those in Group I yield in the 2.5 hr. period distinctly less than 1 atom of N, those in Group II between 1.2 and 1.6 (most of them between 1.24 and 1.35) and those in Group III exactly (or almost exactly) 2. The differing behaviours of these groups are also evident at 30 min., but are either non-existent or (more probably) obscured at 3 min.

Inspection of the lists composing the different groups shows (1) that all the ω -uramido-acids examined belong to the first, (2) that with one exception the α -uramido-acids with straight chain belong to the second and (3) that, again with one exception, the α -uramido-acids with branched chains belong to the third. The exceptions— α -uramidopropionic and α -uramido-*isohexoic* acids (Nos. 2 and 11 in Table 1)—each yield less nitrogen (or react more slowly) than other acids of analogous constitution, so that the first falls into Group I instead of Group II, and the second into Group II instead of Group III. The significance of these apparent anomalies is doubtful; but upon the second of them the following comment is perhaps pertinent. The only compounds which yield in 2.5 hr. the full 2 atoms of N are those (Nos. 12–14), in which the carbon chain branches at either the α - or the β -position. In α -uramido-*isohexoic* acid branching occurs at a point more remote from the uramido group, and the yield of N falls. It is still, however, greater than the 1.3 atoms or so given by the straight-chain aliphatic compounds, Nos. 5–9. This suggests that the speed or completeness of the nitrous acid reaction is affected not merely by the existence, but also by the position, of a branch in the carbon chain, and, more specifically, that proximity of the branch to the uramido group is an accelerating factor. These suggestions are offered with due reserve, for the data prompting them are all too meagre.

Analogy with the amino-acids would lead one to expect that an α -uramido group would react more rapidly than one in any other position. In a general way this expectation is realized, although one departure from the rule is evident in the case of the slowly reacting α -uramidopropionic acid. The analogy with the

amino-acids extends even further. Dunn & Schmidt [1922] have shown that as the distance of the amino group from the carboxyl group increases, the speed of its reaction with nitrous acid continues to diminish. Comparison of ϵ -uramido-hexoic acid (No. 1) with β -uramidopropionic acid (No. 4) and of these with the main group of similarly constituted α -uramido-acids (Nos. 6-9) shows that, generally speaking, this is true of the uramido group also. The fact that the δ -uramido group of citrulline (No. 3) falls partly out of line (for the first hour or so its rate of decomposition is the lowest of all) is probably due to a disturbing influence exerted by its α -amino group or by the hydroxyl group into which the latter is soon converted.

A disturbing influence is in fact evident in each of the two instances in which the uramido group is accompanied by a simple amino group. A mere inspection of the numerical data suffices to show that not only in citrulline (No. 3), but also in its isomeride (No. 8), the behaviour of the uramido group diverges from the general pattern. The form of this divergence becomes clearer, if the data are plotted on a graph. The simple uramido-acids (eleven in number), and even the more complex arginine derivative (No. 5), then yield curves which, though varying in height, have all essentially the same shape. The initial rise is relatively rapid, but it soon slackens, and the later part of the curve is very flat. The curves for the amino-uramido-acids are distinctly different—their earlier parts not so steep, their later less nearly horizontal, their changes of direction accordingly less abrupt. Their whole form is in fact intermediate between that presented by any simple uramido-acid and the gradual yet prolonged upward sweep which would represent our data for urea. The validity of these comparisons depends of course upon the correctness of the assumption inherent in our calculations. The observed results have been interpreted as an effect of the amino group upon the rate of decomposition of the uramido one; the possibility of a converse or reciprocal action has been left out of the reckoning.

It was found by Van Slyke [1929] that the speed with which the α -amino groups of amino-acids react with nitrous acid is dependent not upon temperature alone, but also upon the volume of the mixture in the reaction chamber. It is probable that this is equally true of uramido groups. The results reported in this paper would then be strictly applicable only under the adopted conditions—a temperature of 25° and a volume of 8 ml. The gross differences encountered would doubtless be evident in some degree under any conditions, but the details of the reaction in any individual case could not be expected to be always the same. Our results, although somewhat different from, are not therefore necessarily discordant with, the few previously recorded ones mentioned at the beginning of this paper [Rohde, 1918; Wada, 1930; Hunter, 1938]. Most of these, in any case, were obtained by the original volumetric technique of Van Slyke [1913].

As has been stated, our interest in the problem was purely empirical. We have made no study of the mechanism of the reaction between nitrous acid and the uramido group, or of the mode of operation of the factors which appear to modify its course.

SUMMARY

Data are reported exemplifying the behaviour of various uramido-acids in the nitrous acid method for the determination of amino-nitrogen, and showing that this behaviour is far from uniform. Under certain specified conditions the amount of nitrogen evolved in 2.5 hr. varies from 0.66 to 2 atoms. The observed differences can, to a considerable extent, be related to structural differences in the various acids examined.

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