THE REACTION OF NAPHTHOQUINONE-4-SULFONATE WITH IMINO ACIDS

By WALTER TROLL*

(From the Department of Chemistry, New York University College of Medicine, New York, New York)

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Naphthoquinone-4-sulfonate[1] was introduced by Folin as a reagent for the colorimetric determination of amino acids (1). The main advantage of this procedure of Folin was its simplicity. Colors were developed at room temperature in slightly alkaline solution. The drawback remained that the yield of color varied from amino acid to amino acid. A modification introduced by Frame *et al.* (2) using borate buffer at 100° did not resolve this difficulty.

Seeking a simple rapid method for amino acids, we have reinvestigated these reactions. Although color development by amino acids appeared to be complete in less than 1 hour at room temperature, conditions have not been found for stoichiometric reaction. Since NQS appeared also less specific for amino acids than ninhydrin, the attempt to employ it analytically for amino acids was abandoned. The imino acids proline and hydroxyproline, however, were found to react quantitatively and stoichiometrically. We have been able to isolate their reaction products and devise a method for the determination of total imino acids in protein hydrolysates.

EXPERIMENTAL

Isolation of Products of NQS with Imino Acids and Aromatic Amines

The reaction was carried out in a bicarbonate buffer (pH 8.3) at room temperature with a 5-fold excess of NQS over the imino acid or amine. Aromatic amines and imines precipitated from the reaction mixture. The colored products from the imino acids proline, hydroxyproline, and sarcosine crystallized out on acidifying to pH 3. Excess acid should be avoided, as the pigments are unstable in solutions more acid than pH 2.

The imino acid products could be purified by alternate dissolution in dilute sodium bicarbonate and precipitation by addition of acid. The crystals were slightly soluble in water, methyl, and ethyl alcohol, and

* Present address, May Institute for Medical Research of the Jewish Hospital Association, Cincinnati, Ohio.

[1] The following abbreviations are used: NQS, naphthoquinone-4-sulfonate; NQ, naphthoquinone.

insoluble in ether, chloroform, and hexane. Titration of the crystals with alkali indicated the presence of an acidic group, with a pK close to 4, per nitrogen atom (Table I). This suggested that the carboxyl group of the imino acid was retained in the colored products. The compounds formed by aniline and *N*-methylaniline were recrystallized from acetone. The aniline compound has been shown to be the 4-anil compound I (3) and our nitrogen analyses were in agreement with this formulation (Table I). The equivalent weights calculated from the nitrogen content of the imino acid compounds (Table I) were in agreement with an analogous composi-

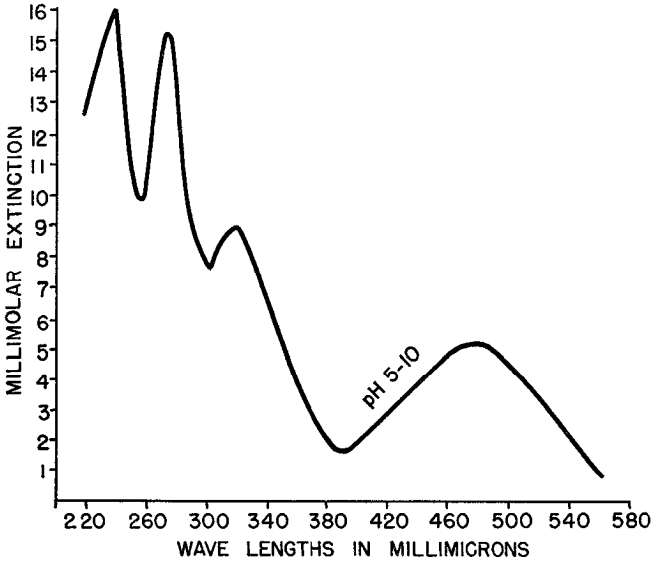


FIG. 1. Absorption spectra of the NQ compounds of the imino acids proline, hydroxyproline, and sarcosine.

tion. The orthoquinoidal structure II is suggested for the NQ-imino compounds on the basis of the observation that their absorption spectra remained unchanged on raising the pH from 5 to 10 (Fig. 1), a behavior indicative of the absence of a phenolic group, whereas in the case of the NQ-anil compound this measure produced a shift of the absorption maximum at 480 mμ towards a lower wave-length (Fig. 2).

Reaction of Imino and Amino Acids with NQS

The imino acids, proline and hydroxyproline, appeared to form the compounds, whose isolation is described above, in a quantitative manner, when allowed to react with NQS in a bicarbonate solution (pH 8.3) at room temperature for 5 minutes. The absorption spectra obtained between 420

and 600 mμ, by allowing a quantity of proline or hydroxyproline to react as described above, were identical, after removal of the NQS color in this

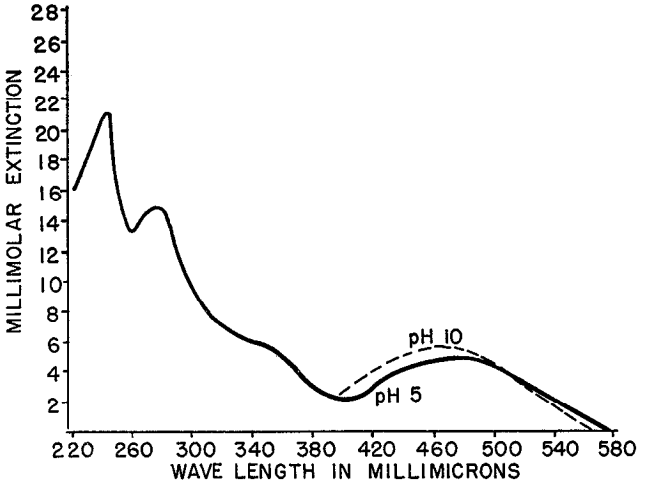
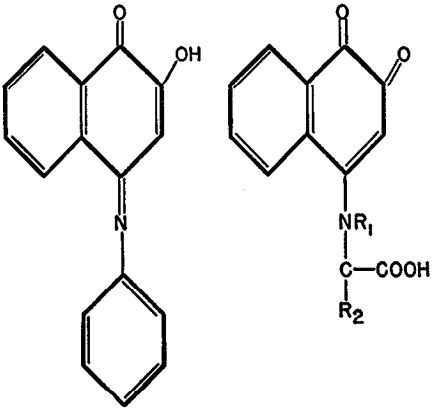


FIG. 2. Absorption spectrum of the NQ compound of aniline

I II



region by reduction with ascorbic acid, with those obtained from dissolving an equimolar quantity of the isolated compound in the same solvent.

Amino acids, except for tryptophan, reacted more slowly and formed a reaction product with a distinctly different absorption spectrum. The most striking difference was the movement of the maximum at 470 mμ to longer wave-lengths when the pH was raised from 5 to 8. Tryptophan, however, behaved like an imino acid in this reaction. It reacted at the

same rate as imino acids and formed a reaction product analogous in composition to the imino acid compounds (Table I) with an absorption spectrum which remained unchanged between pH 5 and 10. No adequate explanation can be given for the exceptional behavior of tryptophan, but it is of interest that it also reacts with formaldehyde like an imino acid, being able to combine with only 1 molecule of formaldehyde, while all other amino acids combine with 2 molecules (4).

Since proline and hydroxyproline reacted quantitatively to form colors which were indistinguishable, this reaction appeared ideally suited to measure the molar sum of these amino acids. The amino acids must first be deaminated by a preliminary treatment with nitrous acid. The resulting

TABLE I

Data of Naphthoquinone-4-Sulfonate Compounds

Compound	Equivalent weight		Theoretical mol. wt.*	Apparent pK of carboxyl	mM extinction coefficient at maximum indicated
	Calculated from nitrogen content	Determined alkalimetrically			
Proline	275	273	271	3.8	5.11 (480 mμ)
Hydroxyproline	288	290	288	3.9	5.12 (480 ")
Sarcosine	245	250	245	3.8	5.11 (480 ")
Tryptophan		362	360	4.2	4.90 (480 ")
Aniline	255		249		4.82 (480 ")
N-Methylaniline	270		263		6.25 (490 ")

* The molecular weights are the ones of the assumed structures I and II.

nitrosamines of proline and hydroxyproline are hydrolyzed and the color is then developed with NQS. A preliminary report of this method has been given (5). Subsequently, Hamilton and Ortiz (6) published a method for imino acids based upon a nitrosation procedure followed by a gasometric ninhydrin determination.

Procedure for Determination of Proline Plus Hydroxyproline

Reagents—

1. 1,2-Naphthoquinone-4-sulfonate. The commercially available preparation (Eastman Kodak) requires two recrystallizations by Folin's borate procedure (1). It can be prepared conveniently from 1-amino-2-hydroxynaphthalene-4-sulfonic acid by oxidation with nitric acid, followed by two recrystallizations (1). A 0.02 M aqueous solution is prepared within 4 hours of use.

2. 1 M sodium bicarbonate.

3. Acetate buffer, pH 5, 1 M.

4. 0.1 M ascorbic acid in 0.001 M hydrochloric acid. This solution is stable for several weeks in the refrigerator.

5. 6 M sodium nitrite.

6. Glacial acetic acid.

7. Concentrated hydrochloric acid (iron-free), c.p., 0.1 N hydrochloric acid.

8. 2 N sodium hydroxide.

Procedure—1 ml. of protein hydrolysate containing from 3 to 10 mg. of hydrolyzed protein and no more than 3 m.eq. of hydrochloric acid is allowed to react with 1 ml. of 6 M sodium nitrite and 0.3 ml. of glacial acetic acid in a 50 ml. Erlenmeyer flask. The flask is allowed to stand for 1 hour with occasional shaking. 10 ml. of concentrated hydrochloric acid and a boiling stone are added and the solution is evaporated to about 2 ml. This procedure, starting with the addition of the concentrated hydrochloric acid, is repeated twice. Then the solution is titrated with 2 N sodium hydroxide to the red end-point of phenolphthalein and then with 0.1 N HCl just to the colorless point. 1 ml. of 1 M sodium bicarbonate, 2 ml. of 0.02 M NQS, and water to bring the total volume to about 10 ml. are added. After 10 minutes, 1 ml. of acetate buffer and 1 ml. of ascorbic acid solution are added and the solution is made up to volume, 25 or 50 ml. The optical density of the solution at 480 mμ is then determined. 1 ml. of the protein hydrolysate is treated identically, except that the addition of NQS is omitted and 1 ml. of water is subjected to the total procedure. The optical densities at 480 mμ obtained from these two determinations are subtracted from the optical density observed in the procedure described above. For standards, either proline or hydroxyproline may be used, since they give identical values on a molar basis.

Results—In recovery experiments, 1 to 5 μM of proline or of hydroxyproline was added to a mixture of amino acids. Recovery of 96 to 99 per cent imino acid nitrogen was achieved. Similar recoveries were obtained when proline was added to a bovine serum albumin hydrolysate (Table II).

The method has been applied to the analysis of lactoglobulin, bovine serum albumin, and gelatin hydrolysates. Hydroxyproline was determined by the ninhydrin method which has been described (7). The results are given in Table III where they are compared with those obtained by other methods.

Creatine forms an interfering color with NQS after nitrous acid treatment. The spectrum of this reaction product was identical with that obtained from imino acid-NQS reaction mixtures. Nitrosated creatine was chromatographed on paper with a 1:1 mixture of benzyl alcohol and phenol

TABLE II

Recovery of Imino Acids from Mixtures

Imino acids added to 1 ml. of a solution containing 0.01 mole per liter of glycine, alanine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, methionine, lysine, and arginine and to 1 ml. of bovine serum albumin hydrolysate.

Imino acid used	Imino acid added	Optical density observed at 480 mμ	Optical density calculated from mM extinction coefficient of imino acid-NQ compound at 480 mμ	Recovery, per cent
Amino acid mixture, final volume 25 ml.				
	μM			
None	None	0.001		
Proline	1.0	0.200	0.204	98
"	2.0	0.396	0.408	97
"	3.0	0.600	0.612	98
Hydroxyproline	4.0	0.801	0.816	98
"	5.0	1.03	1.020	101
Bovine serum albumin (7.59 mg. per ml.), final volume 50 ml.				
			Optical density calculated from mM extinction coefficient of imino acid-NQS compound at 480 mμ + 0.337	
None	None	0.337		
Proline	4.0	0.740	0.745	99
"	5.0	0.830	0.847	98

TABLE III

Results from Protein Hydrolysates

Protein	Observed results		Other methods	
	Proline, gm. per 100 gm. protein	Hydroxyproline, gm. per 100 gm. protein	Proline, gm. per 100 gm. protein	Hydroxyproline, gm. per 100 gm. protein
Lactoglobulin (crystalline), 15.6% N	4.86	0.08 (7)	4.79 (8)* 5.10 (9)	0.05 (10)
Bovine serum albumin (crystalline), 16.07% N	5.02	0.06 (7)	5.07 (10) 4.75 (9)	0.005 (10)
Gelatin	14.0	12.83 (7)	14.8 (11)	13.1 (12)

* Bibliographic reference No.

saturated with water and the paper was sprayed with NQS solution in 0.1 M sodium bicarbonate, after development for 12 hours. A red spot appeared that was identical in its position with that produced by sarcosine when subjected to this chromatographic procedure. Sarcosine mixed with nitrosated creatine produced a single spot, while proline plus nitrosated creatine gave two spots and hydroxyproline, proline, and nitrosated creatine, three spots. It appears, then, that, in the reaction with nitrous acid, a portion of the creatine is transformed to sarcosine.

Because of this interference of creatine, the method described above in its present form cannot be applied to urine or blood. Other urine constituents do not appear to interfere.

SUMMARY

1. Imino acids formed mononaphthoquinone derivatives quantitatively with 1,2-naphthoquinone-4-sulfonate.

2. A method for the determination of imino acids in the presence of amino acids was developed.

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BIBLIOGRAPHY

1. Folin, O., *J. Biol. Chem.*, **51**, 377 (1922).
2. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, **149**, 255 (1943).
3. Boniger, M., *Ber. chem. Ges.*, **21**, 25 (1894).
4. Levy, M., and Silberman, D. E., *J. Biol. Chem.*, **118**, 723 (1937).
5. Troll, W., Presented before the New York section of the Society for Experimental Biology and Medicine, Dec. 14 (1949).
6. Hamilton, P. B., and Ortiz, P. J., *J. Biol. Chem.*, **187**, 733 (1950).
7. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, **200**, 803 (1953).
8. Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, **71**, 249 (1949).
9. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178**, 78 (1949).
10. Keston, A. S., and Udenfriend, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 92 (1949).
11. Brand, E., *Ann. New York Acad. Sc.*, **47**, 213 (1946).
12. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, **184**, 299 (1950).