Colorimetric Analysis of Organic Hydroxyl Groups

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The reaction of 3,5-dinitrobenzoyl chloride with organic hydroxyl groups has been adapted to provide a colorimetric method of analysis. The ester formed by this reaction is subjected to conditions of controlled basicity after hydrolysis of the excess reagent. A red color is formed by the ester which is stable after 30 min. for an additional 20 min. or more, and is measured at 555 mμ *versus* a reagent blank. Tertiary alcohols react too slowly to interfere, and tertiary amines do not react. Phenols, primary and secondary amines, and certain thiols react similarly to hydroxyl groups and can be determined by this method. The procedure is rapid, simple, and accurate.

THE PRODUCTION of color on the addition of base to polynitroaromatics is well known. It has been used as a means of qualitative and quantitative determination of nitroaromatics by Porter (1). Berezin (2) and Robinson, Cundiff, and Markunas (3) have described the use of 3,5-dinitrobenzoyl chloride for the determination of alcohols in which the excess reagent is hydrolyzed and titrated with base. The end point of the titration is indicated by color formation due to the 3,5-dinitrobenzoate ester of the alcohol and the 3,5-dinitrobenzoate anion. Robinson, Cundiff, and Markunas (3) also pointed out that the ester could be determined directly by potentiometric titration since the end point potential is sufficiently removed from that for the anion to allow a differentiating titration.

A number of other excellent methods are available for the titrimetric analysis of hydroxyl compounds on a milligram or larger scale (5, 10–12). At the sensitivity level of colorimetric analyses several methods are available for specific hydroxyl types. As examples, those of Dal Nogare and Mitchell (4), Schreiber and Eschenmoser (5), and Critchfield and Hutchinson (6) may be cited.

However, few generally applicable colorimetric hydroxyl methods are available. The method of Baggett, Engel, and Fielding (7) may be adapted to general use for all hydroxyl compounds which are subject to acetylation. Acetylation is also the basis of a method proposed by Gutnikov and Schenk (8) which utilizes the ferric hydroxamate reaction after acid-catalyzed esterification of the hydroxyl functions. The sequence of reactions involved necessarily makes this a complex procedure, though it has the compensating advantage of allowing analyses of tertiary alcohols and avoids interference by amines. The method of Johnson and Critchfield (9) utilizes the 3,5-D reaction. It also has the advantage of eliminating amine interferences because of an extraction step, but has several disadvantages when compared to the method described here. Satisfactory response was not obtained by these investigators with diols. Larger volumes and more complex treatment are required, and the color produced is not stable for more than 4 min. The method proposed here is markedly superior on each of these points.

The information reported by Robinson, Cundiff, and Markunas (3) indicated the possibility that the 3,5-dinitrobenzoate esters become colored in a system of lower basicity than that required to produce color with 3,5-dinitrobenzoic acid. The problem of capitalizing on this differential color development was then to devise a system wherein the ester was primarily in the colored state, and the acid was not, and to maintain this condition long enough for convenient measurement. The method described here presents an approach based on this concept.

The method involves formation of the 3,5-dinitrobenzoate ester by reaction of the hydroxyl compound under study with a large excess of 3,5-dinitrobenzoyl chloride in pyridine–dimethylformamide. The excess reagent is hydrolyzed, the resulting mixture buffered with a saturated solution of ammonium acetate in acetone, and concentrated ammonia is added. Final dilution is made with acetone. Under these circumstances only faint color is developed with a reagent blank, and is probably due to impurities in the solvents used. The measurement is made at 555 mμ with a suitable spectrophotometer or colorimeter.

EXPERIMENTAL

Reagents and Apparatus—3,5-Dinitrobenzoyl chloride (Eastman White Label reagent), 540 mg., is dissolved in 2 ml. of dry dimethylformamide. This solution is stable for at least 72 hr. A saturated solution of ammonium acetate is prepared in dry acetone at 25° to 29°. The Cary model 11, Beckman DU, and Beckman B spectrophotometers were used in this study.

Procedure—One milliliter of the sample solution (25–300 mcg./ml. in dry pyridine) is transferred to a 10-ml. volumetric flask. One-tenth milliliter of the

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3,5-dinitrobenzoyl chloride solution is added, and the mixture is allowed to stand for 15 min. at room temperature. Approximately 0.25 ml. (5 drops) of water is then added, and hydrolysis of the excess reagent is carried out by heating on a steam bath for 5 min. When the sample has cooled (5 to 15 min.), 2 ml. of the buffer solution and 0.5 ml. of concentrated (15 *N*) ammonium hydroxide reagent are added. After 30 min. the mixture is diluted to 10 ml. with acetone and the absorbance measured at 555 mμ *versus* a similarly prepared reagent blank. The color is stable for at least an additional 20 min.

DISCUSSION

Table I summarizes analytical data obtained for a variety of compound types by this method. In every case, except the tertiary hydroxyl compound (*tert*-butyl alcohol), the response is linear over the normally useful absorbance range. The absorption spectrum as shown in Fig. 1 for ethanol is essentially identical for all compounds listed in the table.

All the listed compounds were determined using the procedure exactly as described above. It is possible that increased reaction time or temperature for the esterification may result in a satisfactory response for tertiary hydroxyls. Careful limitation of reaction time will permit the determination of primary or secondary alcohols in the presence of approximately equivalent amounts of tertiary alcohols (9).

The failure of 2-mercaptobenzothiazole to react may be due to a possible opening of the lactone ring resulting in a nonreactive structure. Mercaptoacetic acid yielded color immediately upon addition of the dinitrobenzoyl chloride reagent. However, this color remained stable throughout the balance of the analytical procedure and presented no difficulties.

The problem of buffering at an appropriate level of basicity to provide reasonable sensitivity without excessive color in the blank solution was most critical. This was dealt with in the following manner.

Table I—Application of the Method

Compd.	Taken, mcg.	Found, mcg.	Error, mcg.
Ethanol	200	203	3
1-Propanol	200	193	7
1-Butanol	200	189	11
2-Butanol	200	204	4
1,3-Propanediol	100	97	3
Testosterone	200	196	4
11α-Hydroxytestosterone	100	101	1
11α-Hydroxyprogesterone	200	202	2
6α-Methyl dienediol[a]	300	294	6
Phenol	278	275	3
n-Butylamine	206	204	2
Aniline	145	143	2
Piperidine	307	309	2
Mercaptoacetic acid	205	210	5
Mercaptoethanol	207	203	4
Mercaptoethyl amine	220	223	3
2-Mercaptobenzothiazole		No reaction	
tert-Butyl alcohol		Slow reaction	

[a] 11β,21-Dihydroxy-6α-methylpregna-4,17(*cis*)-dien-3-one.

With each solvent system and base tested during development of the method, a titration similar to that of Robinson, Cundiff, and Markunas (3) was carried out with appropriate quantities of ethanol

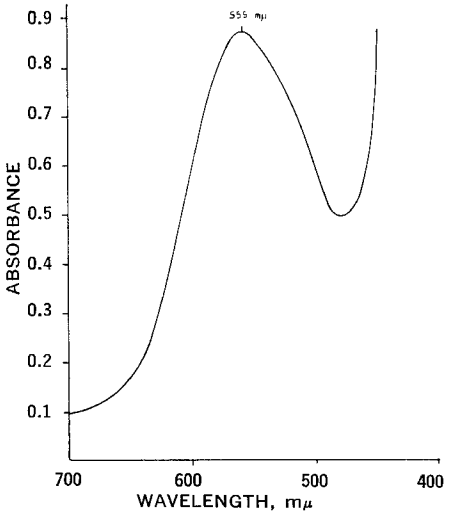

Fig. 1—Absorption spectrum of the 3,5-dinitrobenzoate ester of ethanol (201 mcg. of ethanol in 10 ml. solution vs. reagent blank).

Table II—Analysis of Mixtures

Mixture, Wt. % Each	Sample Taken, mcg. of Ethanol	Sample Recovery, mcg.	Error, mcg.
Ethanol–acetic acid			
0.1–99.9	196	183	13
1.0–99.0	196	193	3
10.0–90.0	196	197	1
Ethanol–acetone			
0.1–99.9	207	214	7
1.0–99.0	207	210	3
10.0–90.0	207	222	15
Ethanol–acetaldehyde			
0.1–99.9	195	207	12
1.0–99.0	195	198	3
10.0–90.0	195	192	3
Ethanol–ethyl acetate			
0.1–99.9	221	237	16
1.0–99.0	221	226	5
10.0–90.0	221	220	1
Ethanol–water			
15.0–85.0	300	305	5
10.0–90.0	300	310	10
7.0–93.0	300	290	10
4.8–95.2	300	270	30
3.6–96.4	300	255	45
3.0–97.0	300	245	55
2.5–97.5	300	235	65
2.1–97.9	300	225	75
Ethanol–concentrated HCl			
15.0–85.0	300	283	17
10.0–90.0	300	287	13
7.0–93.0	300	260	40
4.8–95.2	300	215	85
3.6–96.4	300	167	133

and 3,5-dinitrobenzoyl chloride reagent. The titration was carried out potentiometrically beyond the second break (titration of the benzoate ester) using a glass-saturated calomel electrode system and one of several different bases as titrant. Following this, solid sodium or ammonium acetate was added to determine whether the potential of the solution could be reversed partially and made to stabilize at the beginning of the second break. At this point, color development due to the ester is near a maximum, while color due to the 3,5-dinitrobenzoic acid is negligible. In this manner solvent systems involving pyridine, acetonitrile, dimethylformamide, acetone and chloroform alone, and some selected mixtures, were tested to arrive at the solvent mixture used in the procedure. Tetrabutylammonium hydroxide (1 *M* in methanol) and KOH (1 *M* in water) were tested as bases before turning to concentrated ammonia.

It is obvious that any of the reactive compound types would constitute a positive interference in the analysis of any other. Similarly, reactive acyl functions (anhydrides or halides) will tend to show negative interference depending on the efficiency with which they compete with the 3,5-D reagent for the available hydroxyl groups. The fact that both hydrochloric and 3,5-dinitrobenzoic acids are formed during the esterification reaction indicates that these types of compounds would not constitute interferences if present in small amounts. Water will react preferentially with 3,5-D, but will not interfere seriously unless present in sufficient quantity to consume a large proportion of the available reagent. Additional compounds which have been tested and shown not to interfere seriously in the determination of hydroxyl groups are summarized in the data of Table II. Note also in Table I that the multifunctional steroids imply a lack of interference by unsaturated moieties and by simple and conjugated ketones.

SUMMARY

A method has been presented and described for the colorimetric determination of organic alcohol, amine, and thiol groups. The method is free of interference from most common solvents and other functional groups. The procedure is rapid and the resulting products are adequately stable to provide ease of measurement.

REFERENCES

(1) Porter, C. C., *Anal. Chem.*, **27**, 805(1955).
(2) Berezin, J. V., *Doklady Akad. Nauk, S.S.S.R.*, **99**, 563(1954); through *Chem. Abstr.*, **50**, 859g(1956).
(3) Robinson, W. F., Jr., Cundiff, R. H., and Markunas, P. C., *Anal. Chem.*, **33**, 1030(1961).
(4) Dal Nogare, S., and Mitchell, J., Jr., *ibid.*, **25**, 1376 (1953).
(5) Schreiber, J., and Eschenmoser, A., *Helv. Chim. Acta*, **38**, 1529(1955).
(6) Critchfield, F. E., and Hutchinson, J. A., *Anal. Chem.*, **32**, 862(1960).
(7) Baggett, B., Engel, L. L., and Fielding, L. L., *J. Biol. Chem.*, **213**, 87(1955).
(8) Gutnikov, G., and Schenk, G. H., *Anal. Chem.*, **34**, 1316(1962).
(9) Johnson, D. P., and Critchfield, F. E., *ibid.*, **32**, 865(1960).
(10) Fritz, J. S., and Schenk, G. H., *ibid.*, **31**, 1808(1959).
(11) Schenk, G. H., and Santiago, M., *Microchem. J.*, **6**, 77(1962).
(12) Sully, B. D., *Analyst*, **87**, 940(1962).

Behavior of Erythrocytes in Various Solvent Systems III

Water–Polyethylene Glycols

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Hemolytic behavior of human and rabbit erythrocytes in aqueous solutions of polyethylene glycol (PEG) 200, 300, 400, and 600 was investigated. Complete hemolysis occurred in all PEG 200 and 300 solutions, with discoloration occurring in $\geq$ 25 per cent PEG 200 and $\geq$ 15 per cent PEG 300 solutions. Sodium chloride was effective in preventing hemolysis in $\leq$ 25 per cent PEG 200 or $\leq$ 40 per cent PEG 300 solutions. When possible, *i* values were calculated for sodium chloride in the various water–PEG 200 and 300 solutions. PEG 400 and 600 protected blood cells from damage in > 10 per cent to < 40 per cent solutions, and *i* values were calculated for these PEG's. Solutions containing $\geq$ 40 per cent PEG 400 or 600 (with and without NaCl) were damaging to red cells. The ability of liquid PEG's to penetrate rabbit and human erythrocytes appeared to be 200 > 300 > 400 > 600.

PREVIOUS PAPERS in this series have reported the behavior of erythrocytes in various water–glycerin and water–propylene glycol systems (1, 2). Among other nonaqueous solvents that might be used in the preparation of parenterals would be the liquid polyethylene glycols.

Polyethylene glycols (PEG's) are products possessing a very low order of toxicity. Spiegel and Noseworthy (3) have reviewed the physical properties, toxicities, and parenteral applications of these liquids. Skin penetration studies on

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