The data indicate that 0.5-hour digestions are more than adequate for the destruction of the organic portion of a substance with compounds as refractory as tryptophan and that prolonged digestion can result in the oxidation of appreciable quantities of ammonia. Recently it has been found that several compounds containing quaternary nitrogen groups gave very poor recovery of ammonia nitrogen by the sealed tube method, whereas the conventional Kjeldahl procedure proved satisfactory. A preliminary investigation has shown that the rate of increase of digestion temperature plays an important part in the resulting conversion to ammonia. Further study is being conducted on this problem.

Because the addition of water to the digestion mixture decreases the rate of oxidation of ammonia, temperature control need not be so precise if the digestion mixture contains small amounts of water, and temperatures somewhat greater than 470° C. may be used without loss of ammonia.

A further study of the chemistry of the ammonia oxidation step is being conducted. It is hoped that such a study will lead to a better understanding of the factors which may be controlled in this oxidation.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of A. S. Newton, University of California Radiation Laboratory, who carried out the mass spectrographic analysis reported in this paper. This investigation was supported in part by grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the University of California Committee on Research.

LITERATURE CITED

(1) Baker, P. R. W., *Analyst*, **78**, 500 (1953).
(2) Grunbaum, B. W., Schaffer, F. L., and Kirk, P. L., ANAL. CHEM., **24**, 1487 (1952).
(3) Kirk, P. L., IND. ENG. CHEM., ANAL. ED., **8**, 223 (1936).
(4) White, L. M., and Long, M. C., ANAL. CHEM., **23**, 363 (1951).

RECEIVED for review June 17, 1954. Accepted November 20, 1954.

Determination of Hydroxy and Amino Compounds by a Chlorine-36–Isotope Dilution Method

POUL SORENSEN

Central Laboratory, Sadolin & Holmblad, Ltd., Copenhagen, Denmark

A modification of isotope dilution analysis is proposed by which it is possible to determine hydroxy and amino compounds. The compound to be analyzed is quantitatively converted to a chlorine-containing derivative, and this is determined by an ordinary isotope dilution method. The principle has been applied to determination of phenol, pyrocatechol, methanol, ethylene glycol, aniline, and ethylenediamine.

RADIOACTIVITY measurements can be performed easily and with good precision on chlorine-36 compounds (*5*). Chlorine-containing compounds may thus be determined with a corresponding precision by chlorine-36 dilution methods (*1*, *4*).

In this paper a method is suggested by which non-chlorine-containing compounds may be analyzed by a chlorine-36 dilution method. The compound to be analyzed is quantitatively converted to a chlorine-containing derivative, and this is determined by an ordinary isotope dilution method (*4*).

The principle is a modification of the method of Keston and coworkers for the determination of amino acids (*3*). The amino acids, as radioactive derivatives, are analyzed by a reversed isotope dilution method, which has the advantage that isolation of the desired compound always is possible. However, the impurities present in the mixture are highly radioactive and a very rigorous purification of the compounds is necessary.

By the method here suggested no radioactive impurities will be present. It may sometimes be rather difficult to isolate a given compound, but when it can be done the method may be considered as absolutely specific for the compound in question.

In comparison with ordinary isotope dilution analysis the principle proposed has the following advantages: radioactivity measurements are done with chlorine-36, and therefore good precision is obtainable; with one radioactive compound a whole group of compounds may be analyzed; and the reference compound of the analysis is a derivative of the compound to be analyzed. The derivative is usually much easier to prepare in a pure state than the compound itself.

The disadvantage of the principle is that the compound to be analyzed must be quantitatively converted to a derivative.

For the purpose 3-chloroanisic acid (4-methoxy-3-chlorobenzoic acid) has been shown to be useful. Radioactive 3-chloroanisic acid can be prepared with an acceptably high yield of chlorine-36. Using an excess of 3-chloroanisoyl chloride, hydroxy and amino compounds may be quantitatively chloroanisoylated. The derivatives obtained are crystalline and have sharp melting points.

PREPARATION OF MATERIALS

Anisic Acid (*p*-methoxybenzoic acid). A commercially available product was recrystallized twice from toluene: melting point, 184.0–184.5° C.

3-Chloroanisic Acid. The compound was prepared by chlorination of anisic acid by the method of Hopkins and Chrisholm (*2*). The crude product was crystallized twice from toluene: melting point, 216–217° C.

3-Chloroanisoyl Chloride. A suspension of 175 grams of 3-chloroanisic acid in 250 ml. of purified thionyl chloride was refluxed until a clear solution appeared, and then further for 15 minutes. After removal of the excess of thionyl chloride under diminished pressure the 3-chloroanisoyl chloride was distilled at 1 mm. at 120° to 125° C. Yield, nearly quantitative; melting point, 64° to 65° C. Analysis: calculated for $C_8H_6O_2Cl_2$, saponifiable chlorine, 17.3%; found, 17.4%.

Chloroanisoylation of Hydroxy Compounds. The hydroxy compound and 50% excess of 3-chloroanisoyl chloride dissolved in pyridine were heated to reflux for 20 minutes. After absolute alcohol was added to decompose excess 3-chloroanisoyl chloride, the mixture was poured into water. The precipitate was removed by filtration, washed with water, and crystallized to constant melting point from an organic solvent.

Chloroanisoylation of Amino Compounds. The preparation was the same as described above, except that the reaction mixture was allowed to stand at room temperature for half an hour. If reaction occurs at refluxing temperature, each amino group may be partly dichloroanisoylated.

For monohydroxy and monoamino compounds it may be advantageous to use an excess of the hydroxy or amino compound.

Solvents for crystallizations were dioxane, absolute alcohol, methanol, and ligroine.

Radioactive Compounds. 3-Chloro(36)-anisic Acid. The

method mentioned here (*2*) was used with some modifications. Radioactive elementary chlorine produced from 11.08 grams of active silver chloride (*4*) was absorbed in 120 ml. of 1*N* sodium hydroxide, 3.90 grams of anisic acid were added, and the solution was placed in a closed flask provided with a dropping funnel. The flask was evacuated to a pressure of about 200 mm. and from the dropping funnel 130 ml. of 1*N* nitric acid were forced into the evacuated space by the atmosphere. This was followed by 10 ml. of a 0.5% solution of sodium hydrosulfite to reduce unreacted chlorine. During the procedure the flask was shaken by hand. The precipitated 3-chloro(36)-anisic acid was removed by filtration, dried, and crystallized twice from 75 ml. of toluene. Yield, 3.22 grams; melting point, 216–217° C.

The product was prepared with an activity of 1 microcurie per milliequivalent.

Chlorine-36 was regenerated from chlorine-containing fractions as silver chloride. Yield of 3-chloro(36)-anisic acid of chlorine consumed was about 75%.

Chloro(36)-anisoylation of Monohydroxy and Monoamino Compounds. 3-Chloro(36)-anisic acid was refluxed with 4 to 6 times as much thionyl chloride and excess thionyl chloride was removed as described earlier. The residue of crude 3-chloro-(36)-anisoyl chloride and 100% excess monohydroxy or monoamino compound, dissolved in pyridine, were refluxed for half an hour. The reaction mixture was then poured into water and the crude product obtained was purified by two crystallizations from an organic solvent. The mother liquor from the crystallizations was saponified and some 3-chloro(36)-anisic acid could be regenerated. Yield of chloro(36)-anisoylated compounds based on 3-chloro(36)-anisic acid consumed were 60 to 90%. The melting point of a product differed not more than a few tenths of a degree from that of the pure compound.

Chloro(36)-anisoylation of Dihydroxy and Diamino Compounds. The reaction was first carried out with an excess of hydroxy or amino compound as described above. An excess of inactive 3-chloroanisoyl chloride was then added. After the reaction had finished alcohol was added, the mixture was poured into water, and the product was purified as mentioned above.

APPARATUS AND MEASUREMENTS

Determination of Purity. The purity of a derivative was determined from the melting point (*4*).

The molar melting point depression must be determined for each component to be analyzed.

Radioactivity Measurements. The technique has been described (*5*).

A ratio of two activities was determined with a statistical error corresponding to a standard deviation of 0.6 to 0.7%.

ANALYTICAL PROCEDURES

Reagents. 3-Chloroanisoyl chloride; distilled, melting point, 64–65° C.

3-Chloroanisoyl derivative of compound to be analyzed; pure.

3-Chloro(36)-anisoyl derivative of compound to be analyzed.

Pyridine; reagent grade, dried over barium oxide.

Dioxane; alcohol (99.5 to 99.9%); methanol; ligroine; all of reagent grade.

Preparation of Active Solution. Dissolve an amount of the 3-chloro(36)-anisoyl derivative corresponding to 0.7 microcurie in 100 ml. of dioxane.

Preparation of Standard Sample. Weigh accurately about 200 mg. of the 3-chloroanisoyl derivative (inactive) and 1.5 ml. of active solution. Add dioxane to the mixture until a clear solution is obtained by boiling. Pour into water and recrystallize the product.

Procedure. Weigh in a test tube an amount which is estimated to contain about 1 meq. of the compound to be analyzed. Add 3 ml. of pyridine and about 0.8 gram of 3-chloroanisoyl chloride and shake until the 3-chloroanisoyl chloride has dissolved. If a hydroxy compound is to be analyzed, reflux for 20 minutes; if an amino compound is to be analyzed, allow to stand at room temperature for half an hour. Add 3 ml. of alcohol and boil for a moment. Add 1.5 ml. of active solution, and determine the amount accurately by weighing before and after addition. Heat the mixture to clear solution and pour into 100 ml. of water. Remove crystals by filtration, and wash with water, and finally with 2 ml. of methanol. Crystallize from an appropriate solvent until the melting point differs by less than 1° from that of the pure 3-chloroanisoyl derivative. Determine the purity from the melting point. Measure the specific activity (activity per unit weight) of this final sample as a ratio of the specific activity of the standard sample (correct for background and self-absorption).

Table I. 3-Chloroanisoyl Derivatives

Compound	M.P., ° C.	Formula	Analysis, % Cl Calcd.	Found
Phenol	141.7–142.0	$C_{14}H_{11}O_3Cl$	13.51	13.46
Pyrocatechol[a]	174.4–174.9	$C_{22}H_{16}O_6Cl_2$	15.86	15.82
Methanol	94.0– 94.3	$C_9H_9O_3Cl$	17.69	17.78
Ethylene glycol[a]	186.5–186.7	$C_{18}H_{16}O_6Cl_2$	17.76	17.80
Aniline	169.2–169.5	$C_{14}H_{12}O_2ClN$	13.56	13.63
Ethylenediamine[a]	243.7–244.1	$C_{18}H_{16}O_4Cl_2N_2$	17.85	17.74

[a] Di-(3-chloroanisoyl) derivative.

CALCULATION

$$B = \left(\frac{1}{r} \times \frac{A}{a} \times b + Ay \times \frac{1 - r}{r}\right) \times \frac{P}{100}$$

and

$$\text{Mg. of compound} = \frac{\text{molecular weight of compound} \times B}{\text{molecular weight of chloroanisoyl derivative}}$$

where

$$r = \frac{\text{specific activity of final sample}}{\text{specific activity of standard sample}}$$

A = milligrams of active solution added
B = milligrams of 3-chloroanisoyl derivative present after reaction with 3-chloroanisoyl chloride
a = milligrams of active solution used in preparing standard sample
b = milligrams of 3-chloroanisoyl derivative used in preparing standard sample
y = milligrams of 3-chloroanisoyl derivative per milligram of active solution
P = per cent purity of final sample

EXPERIMENTAL

The method has been applied to the assay of the following compounds:

1. Phenol, analytical grade.
2. Pyrocatechol, C.P., the product was recrystallized twice from benzene, melting point, 104.5–105° C.
3. Methanol, reagent grade, water-free.
4. Ethylene glycol; the product was obtained as a middle fraction by distillation of a technical product. $n_D^{25} = 1.4298$.
5. Aniline, reagent grade.
6. Ethylenediamine; a commercial product was dried with potassium hydroxide and distilled in a vacuum. Titration with 1*N* hydrochloric acid showed a content of 81.9% ethylenediamine (monohydrate 76.9%). The values in Table IX represent the amounts of 100% ethylenediamine calculated from the titration value.

The data concerning the 3-chloroanisoyl derivatives are listed in Table I.

In the experiments the samples for radioactivity measurements were purified until the melting point differed by less than 0.2° from that of the pure substance. The correction due to the purity is then very small and an estimated melting point depression of 0.5° per per cent of impurity was used.

Only in the analysis of methanol was the difference in melting points about 1°; the melting point depression was determined to be 0.5° per per cent content of ethyl-3-chloroanisate.

DETERMINATION OF PHENOL

The derivative was purified by crystallizing from 10 ml. of alcohol, 5 ml. of alcohol, and twice from 10 ml. of methanol.

DETERMINATION OF PYROCATECHOL

The derivative was purified by two crystallizations from 25 ml. of alcohol.

DETERMINATION OF METHANOL

The usual procedure had to be modified. About 3 meq. of methanol were required in the analysis and 1.2 grams of 3-chloro-

anisoyl chloride were added. The derivative was crystallized twice from 5 ml. of methanol and once from 5 ml. of ligroine (Table V).

DETERMINATION OF ETHYLENE GLYCOL

The derivative was crystallized twice from 10 ml. of a dioxane-alcohol mixture (1 to 1).

DETERMINATION OF ANILINE

The derivative was crystallized from 5 ml. of alcohol and twice from 10 ml. of 61% alcohol.

DETERMINATION OF ETHYLENEDIAMINE

The derivative was crystallized from 20 and 10 ml. of alcohol.

DISCUSSION

Experiments with varying excess of 3-chloroanisoyl chloride (Tables III and VIII) show that the chloroanisoylation may be considered to be quantitative. Even moderate amounts of water are permissible (Tables II and IX).

When it is possible to isolate the desired derivative, a content of homologs will not fundamentally disturb the determination. It was usually considered as outside the scope of this investigation to develop methods of purification when homologs were present. However, the methods indicated may often give sufficient purification (Tables II and VII).

Table II. Determination of Phenol

Phenol, Mg.	Recovered Mg.	Recovered %
103.8	103.5	99.7
103.5	103.5	100.0
103.8	103.9	100.1
100.8	101.8	101.0
101.2[a]	101.9	100.7
103.2[a]	103.3	100.1
103.5[a]	104.0	100.5
104.6[b]	105.3	100.7
104.6[c]	104.7	100.1
104.4[c]	103.7	99.3
104.0[d]	98.1	94.3

[a] 5 mg. of *o*-, 5 mg. of *m*-, and 5 mg. of *p*-cresol added to sample.
[b] 10 mg. of water added to sample.
[c] 25 mg. of water added to sample.
[d] 50 mg. of water added to sample.

Table III. Effect of Varying Excess of 3-Chloroanisoyl Chloride on Determination of Phenol

Phenol, Mg.	3-Chloroanisoyl Chloride, Mg.	Recovered Mg.	Recovered %
100.9	400	100.2	99.3
102.4	600	103.1	100.7
104.2	600	104.4	100.2

Table IV. Determination of Pyrocatechol

Pyrocatechol, Mg.	Recovered Mg.	Recovered %
64.9	64.0	98.6
63.5	63.2	99.5
64.3	63.9	99.4
65.3	64.4	98.6

Table V. Determination of Methanol

Methanol, Mg.	Recovered Mg.	Recovered %
97.2	96.5	99.3
95.6	97.3	101.8
95.6	95.6	100.0
96.7	96.5	99.8
96.6	97.2	100.6

Table VI. Determination of Ethylene Glycol

Ethylene Glycol, Mg.	Recovered Mg.	Recovered %
39.8	39.3	98.5
39.0	38.3	98.2
40.6	40.4	99.5
39.2	38.7	98.7

Table VII. Determination of Aniline

Aniline, Mg.	Recovered Mg.	Recovered %
94.8	95.1	100.3
98.5[a]	98.0	99.5
98.0[a]	97.8	99.8
99.1[a]	98.0	98.9
98.4[a]	97.5	99.1

[a] To the sample were added 5 mg. of *o*- and 5 mg. of *p*-toluidine.

Table VIII. Effect of Varying Excess of 3-Chloroanisoyl Chloride on Determination of Aniline

Aniline, Mg.	3-Chloroanisoyl Chloride, Mg.	Recovered Mg.	Recovered %
97.2	300	97.2	100.0
94.7	400	95.0	100.3
96.0	600	96.1	100.1

Table IX. Determination of Ethylenediamine

Ethylenediamine, Mg.	Recovered Mg.	Recovered %
24.95	23.5	94.2
25.35	23.7	93.5
24.85	23.7	95.4
25.15	24.0	95.4
25.05[a]	23.9	95.4
26.40[a]	24.6	92.8
25.60[b]	21.0	82.2
26.05[b]	21.4	82.1

[a] 10 mg. of water added to sample.
[b] 25 mg. of water added to sample.

In some of the series (Tables IV, VI, and IX) values a little too low are found. It seems reasonable to suppose that the compounds analyzed were not pure.

The precision of the final result will usually be determined mainly by the precision of the radioactivity measurements.

ACKNOWLEDGMENT

The author wishes to thank Sadolin and Holmblad, Ltd., for permission to publish this article. Thanks are due to Jytte Jørn-Jensen for helpful assistance.

LITERATURE CITED

(1) Craig, J. T., Tryon, P. F., and Brown, W. G., ANAL. CHEM., **21**, 1661 (1953).
(2) Hopkins, C. Y., and Chrisholm, M., *Can. J. Research*, **B24**, 208 (1946).
(3) Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, **71**, 249 (1949).
(4) Sorensen, P., ANAL. CHEM., **26**, 1581 (1954).
(5) *Ibid.*, **27**, 391 (1955).

RECEIVED for review July 19, 1954. Accepted November 29, 1954.

Corrections

In the article on "Automatic Titrating and Recording Apparatus for Microbiological Assays" [Eades, C. H., Jr., McKay, B. P., Romans, W. E., and Ruffin, G. P., ANAL. CHEM., **27**, 123 (1955)] reference (9) should read: McKay, B. P., and Eades, C. H., Jr., *Ibid.*, **27**, 123 (1955). In the article on "Electromagnetic Laboratory Valve" [McKay, B. P., and Eades, C. H., Jr., ANAL. CHEM., **27**, 163 (1955)] the reference given in the fourth line of the first paragraph should read: Eades, C. H., Jr., McKay, B. P., Romans, W. E., and Ruffin, G. P., ANAL. CHEM., **27**, 123 (1955).

In the article on "Potentiometric Titration of Very Weak Acids [Deal, V. Z., and Wyld, G. E. A., ANAL. CHEM., **27**, 47 (1955)] the second sentence under Solvent Effect on page 48 should read: "Carboxylic acids, on the other hand, titrate as moderately weak acids in water, as weak acids in dimethyl formamide, and as strong acids in ethylenediamine."