

167. Tsutomu Momose, Yosuke Ohkura, Kazuya Kohashi, and Reiko Nagata :
Organic Analysis. XLV.*¹ Determination of Acetone and
Acetoacetic Acid in Blood with Trinitrobenzene.

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Several reagents have been reported in the literature for the colorimetric determination of acetone bodies in biological materials. These include 2,4-dinitrophenylhydrazine,¹⁾ salicylaldehyde,²⁾ *o*-nitrobenzaldehyde,³⁾ vanillin,⁴⁾ furfural,⁵⁾ and *p*-dimethylaminobenzaldehyde.⁶⁾ The first reagent is sensitive in the color development, and its modified methods^{7,8)} are still useful for the estimation of acetone and acetoacetic acid in blood. The procedures, however, are usually very complicated for the reagent, and may be unsuitable for the routine work in a clinical laboratory.

In the previous papers^{9,10)} of this series, the color reaction of trinitrobenzene with active methylene compounds was improved, and successfully used for the determination of cardiac glycosides which contained unsaturated lactone in their molecule. This paper extends the new methods to the determination of acetone and acetoacetic acid in blood with a simple procedure and a minimum amount of the sample.

Experimental

Reagents

Trinitrobenzene Solution—100 mg. of pure 1,3,5-trinitrobenzene⁹⁾ is dissolved in a mixture of 40 ml. of freshly distilled HCON(CH₃)₂ and 1.5 ml. of 1% aq. HCl, diluted with H₂O to measure 100 ml., and stored under protection from light.

Sodium Hydroxide Solution—9.0 g. of NaOH is dissolved in H₂O, made up to 500 ml., stored in a bottle to which an automatic burette (10 ml.) is attached, and protected from carbon dioxide.

Sodium Dihydrogen Phosphate Solution—15 g. of NaH₂PO₄·2H₂O is dissolved in H₂O to make 500 ml.

For the deproteinization of blood or serum, Somogyi's reagent¹¹⁾ is still useful in the present purpose, but the following reagents¹²⁾ are preferable in the simplicity of preparation and use.

Sodium Tungstate Solution—10.0 g. of Na₂WO₄·2H₂O is dissolved in H₂O, and made up to 100 ml.

Alum Solution—9.6 g. of KAl(SO₄)₂·12H₂O is dissolved in H₂O, and made up to 100 ml.

Special Equipments

Glass-stoppered centrifuge tubes, 12 ml., or usual centrifuge tubes and aluminum foil to cover. Developing test-tubes of about 16×160 mm., on which 10.0 ml. is marked. Heating basket.¹³⁾ Water bath regulated at 37±0.2°.

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Procedure

0.100 ml. of blood is haemolysed with 2.90 ml. of H₂O in a test-tube, and 0.50 ml. of sodium tungstate solution is added. To the mixture, 0.50 ml. of alum solution is added, and mixed well. The mixture is then transferred into a centrifuge tube and centrifuged for 5 min. at 3000 r.p.m.

2.00 ml. of the supernatant clear solution is pipetted into a developing test-tube, and 1.00 ml. of trinitrobenzene solution and 1.00 ml. of NaOH solution are successively added under cooling in an ice-water bath. At the same time, a reagent blank is prepared by adding the color developing agent in the same way to 2.00 ml. of a supernatant solution which is prepared by centrifuging a mixture of 3.00 ml. of H₂O, 0.50 ml. of sodium tungstate solution, and 0.50 ml. of alum solution.

The test-tubes are packed in a heating basket, and dipped in a water bath of 37° for 1 hr. to develop the color under protection from light. After cooling in ice-water, each 4 ml. of NaH₂PO₄ solution is added to all tubes, and heated in a boiling water bath for 15 min. to destroy the other color than these of Me₂CO and acetoacetic acid. The tubes are then cooled in running water, and diluted with NaH₂PO₄ solution to the 10.0 ml. mark on the tubes. The absorption intensity of the sample is measured at 480 m μ with the reagent blank, and the value of Me₂CO and acetoacetic acid is read from the calibration curve which is drawn up below.

Calibration Curve

1.00 g. of purified Me₂CO is dissolved in H₂O and made up to 1000 ml. 100 ml. of this solution is diluted with H₂O to measure 1000 ml. The resulting solution contains 100 γ /ml. of Me₂CO, and then so rediluted to give 0.5, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0 γ /ml. solutions.

Three aliquots of each solution are pipetted into developing test-tubes, 1.00 ml. of trinitrobenzene solution and 1.00 ml. of NaOH solution are successively added, and developed as described under procedure. At the same time, three aliquots of 2.00 ml. of H₂O are treated in the same way for blanks.

When open centrifuge tubes are used in the procedure, three aliquots of 4.00 ml. of each Me₂CO solution are centrifuged for 5 min. with aluminum foil cover, and 2.00 ml. of the solutions are developed as described above to reduce the error caused by evaporation. The absorption intensities are measured with the reagent blank which is prepared by mixing three aliquots of the blanks. The calibration curve thus drawn up is shown in Fig. 1. The value of Me₂CO and acetoacetic acid in blood calculated in mg./dl. is obtained by multiplying the number of γ /ml. by 4.

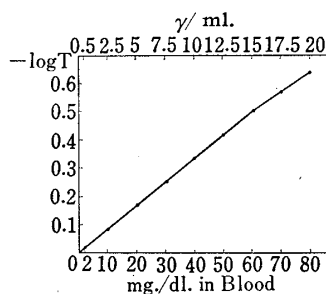


Fig. 1.
Calibration Curve

Results and Discussion

Acetone gives an orange red coloration with trinitrobenzene and sodium hydroxide which has the absorption maximum at 480 m μ . The reagent blank has also a red color, and strongly absorbs around 400~450 m μ as shown in Fig. 2. Therefore, this reaction alone is not suitable for the determination. When an appropriate amount of sodium dihydrogen phosphate is added to the reaction mixture, the blank color diminishes, and the developed color increases without changing the absorption maximum (Fig. 2).

The concentration of sodium hydroxide affects the color development as shown in Fig. 3, and 1.8% solution gives the maximum intensity. The amount of sodium dihydrogen phosphate needed to stabilize the color is shown in Fig. 4, and 2.5% solution may be suitable to dilute the alkaline mixture.

The reaction temperature and time also affect the color development. A higher temperature and prolonged reaction time give a stronger intensity in the color and a deeper blank, but the selected conditions in procedure are preferable because of easiness of operation and constant intensity of the blank color.

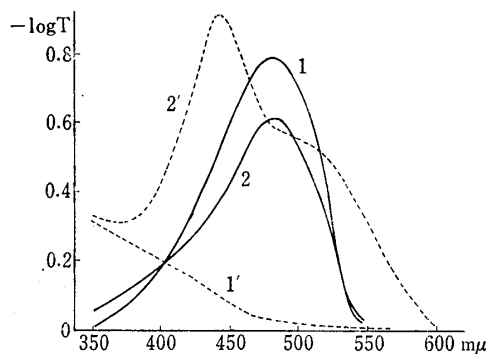


Fig 2. Absorption Curves for Developed and Blank Solutions

2.00 ml. of acetone solution (20 γ /ml.) was warmed with 1.00 ml. of trinitrobenzene solution and 1.00 ml. of NaOH solution for 1 hr. at 37°, and diluted to 10.00 ml. 1: with 6.00 ml. of sodium dihydrogen phosphate solution and 2: with 6.00 ml. of H₂O. 1' and 2' were the corresponding blanks.

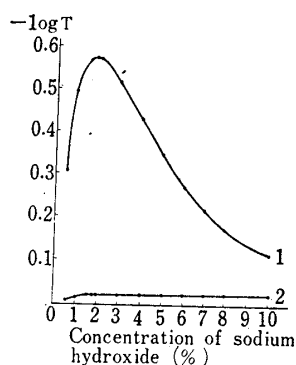


Fig. 3. Effect of Sodium Hydroxide Concentration in the Color Development

- 1: 1.00 ml. of acetone solution (50 γ /ml.) was warmed with 0.50 ml. of trinitrobenzene solution and 0.50 ml. of NaOH solution for 30 min. at 37°, diluted to 10.00 ml. with 6.00 ml. of sodium dihydrogen phosphate solution, and read at 480 m μ .
- 2: 1.00 ml. of H₂O was treated as above for the blanks.

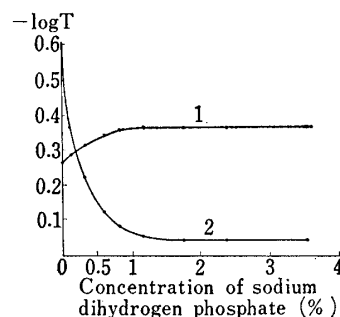


Fig. 4. Effect of Sodium Dihydrogen Phosphate Concentration on the Final Color Intensity

- 1: 1.00 ml. of acetone solution (10 γ /ml.) was warmed with 0.50 ml. of trinitrobenzene solution and 0.50 ml. of NaOH solution for 1 hr. at 37°, diluted to 5.00 ml. with sodium dihydrogen phosphate solution, and read at 480 m μ .
- 2: 1.00 ml. of H₂O was treated as above for the blanks.

Trinitrobenzene solution in the previous paper⁹⁾ was slightly modified by adding a small amount of hydrochloric acid to neutralize basic substances in dimethylformamide. The new solution was stable enough for several months when stored under protection from light.

Acetoacetic acid was quantitatively changed to acetone in the reaction, and gave the same calibration curve as acetone calculated. Therefore, the sum of them was expressed as acetone in this study.

TABLE I. Parallel Tests of Acetone Values on Deproteinizing Agents (Acetone values are expressed in mg./dl.)

Blood No.	Somgyi's agent	Sodium tungstate and alum	Blood No.	Somgyi's agent	Sodium tungstate and alum
1	2.3	2.7	9	12.6	12.6
2	21.6	21.1	10	11.4	11.7
3	2.5	2.5	11	0.7	0.9
4	2.7	2.5	12	1.8	1.8
5	1.8	1.9	13	1.8	1.6
6	59.0	59.5	14	6.5	6.4
7	29.4	29.4	15	2.4	2.3
8	2.0	2.4	16	16.4	16.1

Deproteinization of blood was successfully carried out with Somogyi's agent, or more conveniently with the new agent, sodium tungstate and alum. The data of parallel tests with both deproteinizing agents are shown in Table I, and agree with each other in an experimental error.

Recovery tests were carried out by adding known amounts of acetone and sodium acetoacetate in blood solutions. The results are shown in Table II, which shows an average recovery of 98.5%.

Other substances than active methylene compounds tested which might exist in blood gave no influence on the color development. These included lactic acid, 3-hydroxybutyric acid, creatine, urea and uric acid. Active methylene compounds such as acetaldehyde, pyruvic acid, ascorbic acid, 2-ketoglutaric acid, creatinine, androsterone, glucose and fructose gave a weak coloration with trinitrobenzene and sodium hydroxide. But this influence could be minimized to an extent of experimental error by heating the developed solution in a boiling water bath for 15 minutes except for diabetic blood, to which a correction of acetone value was recommended by subtracting 0.5 mg./dl. of acetone per 100 mg./dl. of glucose. Some preservatives or anticoagulants which might be added in blood gave no influence upon the acetone value in their usual amounts. Those were sodium citrate, sodium oxalate and EDTA. Sodium fluoride gave

TABLE II. Examination of Known Blood Acetone Values

Blood No.	Initial blood acetone and acetoacetic acid value (mg./dl. as acetone)	Acetone or acetoacetic acid added (mg./dl. as acetone)	Total acetone calculated (mg./dl.)	Total acetone found (mg./dl.)	
1	1.3	acetone added	10	11.3	11.7
			20	21.3	21.2
			30	31.3	30.8
			40	41.3	40.5
			50	51.3	50.0
			60	61.3	61.2
			70	71.3	68.6
			80	81.3	80.5
2	1.5	acetone added	10	11.5	11.5
			20	21.5	20.7
			30	31.5	31.1
			40	41.5	40.8
			50	51.5	50.3
			60	61.5	61.8
			70	71.5	70.1
3	2.1	acetoacetic acid added	7.1	9.3	8.9
			14.2	16.3	16.2
			21.3	23.4	23.0
			28.4	30.5	30.2
			35.5	37.6	37.0

TABLE III. Parallel Tests with a 2,4-Dinitrophenylhydrazine Method

Blood No.	Present method (mg./dl.)	2,4-Dinitrophenylhydrazine method (mg./dl.)	Blood No.	Present method (mg./dl.)	2,4-Dinitrophenylhydrazine method (mg./dl.)
1	1.7	1.7	9	17.7	17.4
2	29.4	29.4	10	2.3	2.7
3	2.7	2.4	11	21.6	21.9
4	2.9	2.3	12	2.5	2.5
5	5.4	5.6	13	21.5	21.2
6	6.6	6.7	14	2.7	2.7
7	7.9	7.4	15	1.8	1.8
8	12.3	13.5			

a little larger value with the new deproteinizing agent, which might form $[AlF_6]^{3-}$ with the reagent and hence slight turbidity in the alkaline medium.

The result of parallel tests on acetone and acetoacetic acid in blood with 2,4-dinitrophenylhydrazine method⁸⁾ is shown in Table III. All data well agreed in both methods. The precision of the present method was examined by carrying out 40 separate analysis on two blood solutions. The standard deviation was 0.25 mg./dl. (4.2%) for a mean acetone value of 5.9 mg./dl., and 0.39 mg./dl. (2.4%) for a mean value of 16.5 mg./dl.

This method of determination is simple to treat with a large number of samples, gives reliable results, and may be suitable for a clinical laboratory.

The authors are indebted to Messrs. H. Matsui and M. Hikita, and Miss Y. Soeda for the spectral measurements.

Summary

The sum of acetone and acetoacetic acid in blood was successfully determined with trinitrobenzene as the color developing agent. The method is simple to carry out with a large number of samples, and gives reproducible results.

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168. Masao Nishikawa : Infrared Spectra of Thiourea and its Inclusion Compounds. III.*¹ Conformational Studies of Halocyclohexanes Trapped in Thiourea.

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In the preceding paper of this series,*¹ description was made on the establishment of a new method of studying infrared dichroism of various molecules trapped in thiourea inclusion compounds. In that method, single-crystals of thiourea adducts, about 2 mm. long and 50 μ in diameter, were arranged side by side on a thin sodium chloride plate and their infrared spectra were measured with polarized radiation by the use of an infrared microscope attachment. It was suggested*¹ that the physical state of guest molecules in thiourea channels could be investigated by this technique, and several simple examples were shown there. In the present study, the conformations of halocyclohexane molecules trapped in thiourea channels have been studied, and an outline of the results was reported as a communication.¹⁾ It is well known that halocyclohexanes exist in equilibrium between two chair conformations either with an equatorial or an axial C-X bond. The abundance ratio or equilibrium constant of these two conformations can be best determined by nuclear magnetic resonance, infrared and other physicochemical methods and numerous investigations concerning this

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