Acetate

Frank Lundquist

Very small amounts of acetate are only determined on rare occasions because of the lack of specificity of the methods. The acetate concentration in animal tissues is normally too low to be estimated with the available chemical methods. Enzymatic methods have been described[1-4]. The method described here was developed by *Lundquist, Fugmann* and *Rasmussen*[5].

Principle

An enzyme preparation from pigeon liver catalyses the reactions:

(1) Acetate + ATP ⟶ acetyl-AMP + pyrophosphate

(2) Acetyl-AMP + CoA ⟶ acetyl-CoA + AMP

(3) Acetyl-CoA + sulphanilamide ⟶ acetylsulphanilamide + CoA

The colorimetric determination of the consumption of sulphanilamide in reaction (3) is a measure of the acetate present. Under favourable conditions an 85% yield of acetylsulphanilamide is obtained.

To estimate the very low concentrations of acetate which occur in blood and tissue, and to avoid interference from other compounds (see p. 301), the sample is concentrated before the enzymatic reaction by diffusion in *Conway* units. In most cases, this is also necessary because of the sensitivity of the enzymes to the alkali metal ions.

Reagents

1. Sodium sulphate *)
   anhydrous, acetate-free, A. R.; just before use mix in a mortar with about 5% $Na_2SO_4 \cdot 10\ H_2O$.
2. Perchloric acid, A. R.; sp. gr. 1.67, *ca.* 70% (w/w)
3. Potassium hydroxide, 0.033 N
4. Sodium hydroxide, 1 N
5. Hydrochloric acid, 0.1 N and 0.9 N
6. Adenosine triphosphate, ATP
   crystalline disodium salt, ATP $Na_2H_2 \cdot 3\ H_2O$; commercial preparation, see p. 1006.
7. Coenzyme A (SH-form)
   purity 70–80%; commercial preparation, see p. 1007.
8. Sulphanilamide
9. Potassium citrate, A. R., acetate-free, $K_3C_6H_5O_7 \cdot H_2O$
10. Citric acid, crystalline monohydrate
11. Magnesium chloride, A. R., $MgCl_2 \cdot 6\ H_2O$

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*) The only preparation which could be used directly was that of E. Merck, Darmstadt, Germany. Preparations of other manufacturers must be freed from acetate by steam-distillation of a saturated aqueous solution.

1) *M. Soodak* and *F. Lipmann*, Fed. Proc. *7*, 190 [1948].
2) *R. W. v. Korff*, J. biol. Chemistry *210*, 539 [1954].
3) *I. A. Rose* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 591.
4) *U. Fugmann, F. Lundquist* and *H. Rasmussen:* IV. Internat. Congress for Biochemistry, Vienna 1958, Abstracts. Pergamon Press, London 1958, p. 175.
5) *F. Lundquist, U. Fugmann* and *H. Rasmussen*, Biochem. J. *80*, 393 [1961].

12. Cysteine hydrochloride
13. Tris-hydroxymethyl-aminomethane, tris, A. R.
14. Trichloroacetic acid
15. Sodium nitrite, A. R.
16. Acetic acid
17. Sulphamic acid, H_2NSO_3H
18. *N*-Naphthylethylenediamine dihydrochloride
19. Enzyme preparation
    prepared from pigeon liver according to[6]. The amount of enzyme which brings about a maximum conversion of acetate must be determined for each new enzyme preparation. Preparations obtained according to[6] contain no interfering enzyme contaminants.

Preparation of Solutions

I. Perchloric acid (5% w/v):
Dilute 4.3 ml. 70% (w/w) $HClO_4$ to 100 ml. with distilled water.

II. Sodium hydroxide (2.3 M)-citrate (0.5 M):
Dissolve 9.2 g. NaOH and 10.5 g. citric acid monohydrate in distilled water and make up to 100 ml.

III. Citrate buffer (0.5 M; pH 3.0):
Dissolve 10.5 g. citric acid monohydrate in a little distilled water, adjust to pH 3.0 (glass electrode) with 1 N NaOH (reagent 4.) and dilute to 100 ml. with distilled water.

IV. Coenzyme mixture (sufficient for 200 assays):
Dissolve 300 mg. ATP-$Na_2H_2 \cdot 3\ H_2O$ and 5 mg. coenzyme A in 5 ml. distilled water. Add 3.0 ml. 0.02 M sulphanilamide (172 mg. in 50 ml. distilled water), 2.0 ml. 1 M potassium citrate (16.2 g. $K_3C_6H_5O_7 \cdot H_2O$ in 50 ml. distilled water) and 0.5 ml. 0.1 M magnesium chloride solution (VII).

V. Cysteine hydrochloride (0.5 M):
Dissolve 1.57 g. cysteine hydrochloride in distilled water and make up to 20 ml.

VI. Tris buffer (1.0 M; pH 8.1):
Dissolve 12.1 g. tris-hydroxymethyl-aminomethane in about 80 ml. distilled water, adjust to pH 8.1 (glass electrode) with *ca.* 10 ml. 5 N HCl and dilute to 100 ml. with distilled water.

VII. Magnesium chloride (0.1 M):
Dissolve 2.03 g. $MgCl_2 \cdot 6\ H_2O$ in distilled water and make up to 100 ml.

VIII. Acetic acid standard (17.5 mM):
Dilute 100 µl. acetic acid to 100 ml. with distilled water.

IX. Trichloroacetic acid (5% w/v):
Dissolve 50 g. trichloroacetic acid in distilled water and make up to 1000 ml.

X. Sodium nitrite (0.1% w/v):
Dissolve 200 mg. $NaNO_2$ in distilled water and make up to 200 ml. Prepare afresh daily.

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[6] *M. Soodak* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 266.

XI. Sulphamic acid (0.5% w/v):
Dissolve 1 g. sulphamic acid in distilled water and make up to 200 ml.

XII. *N*-Naphthylethylenediamine dihydrochloride (0.1% w/v):
Dissolve 200 mg. *N*-naphthylethylenediamine dihydrochloride in distilled water and make up to 200 ml.

XIII. Enzyme solution
Immediately before use, thaw the frozen solution prepared according to[6].

Stability of the solutions

The "coenzyme mixture" (solution IV) should be stored in sealed glass ampoules, and in this state it is stable for at least two months at −20° C. The other solutions should be stored at 4° C. Prepare the cysteine and naphthylethylenediamine solution freshly each month. The enzyme solution should be stored in glass ampoules at −20° C and is still usable after a year.

Procedure

The determination is arranged in four steps: 1. deproteinization, 2. diffusion in *Conway* units, 3. enzymatic reaction, 4. sulphanilamide reaction. As the enzymatic reaction does not go to completion it is necessary to include acetate standards in each analysis. A reagent blank is also necessary.

Experimental material

The method has only been tried on tissue homogenates and plasma, but it is certain that it can be used directly or with slight modification for other biological material.

Blank and standards, standard curve

Blank: Neutralize one volume of perchloric acid solution (I) with 1 N NaOH, dilute to 2 volumes with distilled water and add 0.1 volume of citrate buffer (solution III). Carry this "blank mixture" through the complete procedure. If the reagents are sufficiently pure, the optical density of the blank mixture incubated with enzyme is only 0.020 − 0.030 lower than the optical density of an untreated blank mixture which corresponds to the initial sulphanilamide content.

Standard (prepared for each assay): A blank mixture containing acetate is used: add 50 µl. standard acetate solution (VIII) to 10 ml. blank mixture. 2 ml. of this solution corresponds to 10.5 µg. acetic acid.

Standard curve: There is a linear relationship between the concentration and optical density difference up to about 15 µg. acetate/assay mixture. If the acetate concentration is higher, then the deproteinized sample must be diluted correspondingly. Standard curves with 4 − 5 concentrations of acetate, for example, 4, 8, 12, 14 and 16 µg. acetate/assay mixture, should be prepared every month. For this, the diffusion step can be omitted.

Deproteinization

Perchloric acid is best for deproteinization. Metaphosphoric acid has also proved successful. Thoroughly mix plasma with 1 volume of perchloric acid solution (I) and centrifuge after 10 min. Adjust 2 ml. supernatant to pH 3 with 0.2 ml. NaOH-citrate solution (II). Supernatants from other samples must be neutralized with NaOH and then brought to pH 3 by the addition of citrate buffer (solution III). The supernatants should be stored in the cold.

Diffusion

Prepare a *Conway* unit No. 1 [7] with about 8–10 g. solid sodium sulphate (reagent 1.) in the outer ring. Lightly compress the salt with a Perspex pestle shaped in the form of a ring. Place in the centre of the *Conway* unit a cylindrical Perspex dish *) containing 0.6 ml. 0.033 N KOH (reagent 3.). Cool the *Conway* units to 4°C. Pipette 2 ml. of the cold, neutralized extract (pH 3) onto the sodium sulphate layer. Seal **) the *Conway* units and allow to stand for approximately one hour at about 4°C to accelerate the crystallization of the sodium sulphate. The diffusion is complete after allowing to stand for 24 hours at room temperature. Remove the plastic dishes and dry them for half an hour at 80–90°C.

Enzymatic reaction

For the pipetting use *Carlsberg* capillary pipettes. Immediately before use prepare the following incubation mixture:

2 volumes coenzyme mixture (solution IV)
6 volumes tris buffer (solution VI)
1 volume cysteine solution (V)
1 volume $MgCl_2$ solution (VII).

Mix thoroughly. Pipette into each plastic dish:

200 µl. 0.1 N HCl (reagent 4.)
250 µl. incubation mixture.

Mix the contents of the dishes thoroughly, remove

400 µl.

from each dish and transfer to a glass-stoppered centrifuge tube. To this add

150–250 µl. enzyme solution (XIII).

Blow nitrogen through the solutions for 20 sec. and incubate for 1 hour at 37°C. Deproteinize with

2.00 ml. cold trichloroacetic acid solution (IX)

and centrifuge for 10 min.

Sulphanilamide determination

The unreacted sulphanilamide is determined according to *Bratton* and *Marshall* [8]. All the pipettings must be very accurate. It is best to use syringe pipettes.
Pipette into a test tube:

9 ml. 0.9 N HCl (reagent 4.).
1 ml. deproteinized supernatant from the enzymatic reaction
1 ml. sodium nitrite solution (X).

Mix and after 2–3 min. (for destruction of the excess nitrite) add

1 ml. sulphamic acid solution (XI).

*) The dimensions of these dishes are: external diameter: 32 mm., internal diameter: 29–30 mm., external height: 5 mm., internal height: 4 mm. The dishes are turned from Perspex rod.

**) A mixture of 2 parts solid paraffin and 3 parts paraffin oil is used as adhesive and it is applied with a syringe.

7) *E. J. Conway:* Microdiffusion Analysis. 4th edition, Crosby, Lockwood and Son, Ltd., London 1957.

8) *A. C. Bratton* and *E. K. J. Marshall*, J. biol. Chemistry *128*, 537 [1939].

Shake vigorously to facilitate the evolution of nitrogen and after a few minutes pipette in

1 ml. *N*-naphthylethylenediamine reagent (XII).

Measure the optical density of the magenta solutions at 540 mμ.

Calculations

As the standard solutions are treated in exactly the same way as the unknown samples, the amount of acetate contained in 2 ml. of the deproteinized supernatant is:

$$\frac{\Delta E_u}{\Delta E_s} \times 10.5 = \mu g. \text{ acetate}$$

where

$\Delta E_u = E_{blank} - E_{sample}$

$\Delta E_s = E_{blank} - E_{standard}$

10.5 = μg. acetate in 2 ml. standard solution.

The acetate concentration in plasma is

$$\frac{2.11}{2.00} \times \frac{\Delta E_u}{\Delta E_s} \times 10.5 \approx 11 \times \frac{\Delta E_u}{\Delta E_s} = \mu g. \text{ acetate/ml. plasma}$$

(assuming that plasma contains 8% solids). The calculations are similar for other biological material.

Sources of Error

According to *Soodak*[6] pyruvate and formate do not interfere. Propionate gives about 0.7%, butyrate 1.3% and valerate 0.4% of the colour change obtained with an equivalent amount of acetate. Therefore these substances need not be considered as likely sources of error. Acetoacetate reacts with the pigeon liver enzyme, but when the diffusion technique is used acetoacetate does not interfere with the acetate estimation, even if present in a concentration higher than 100 mg./100 ml. plasma. Acetyl-CoA is relatively stable and therefore it does not react in this method[9]. On the other hand, acetyladenylic acid is labile and is rapidly hydrolysed at pH < 2, so if present in the sample it is determined as free acetate[10].

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[9] *E. R. Stadtman* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 931.

[10] *P. Berg*, J. biol. Chemistry *222*, 1015 [1956].