

presence of potassium, ammonium, or rubidium ions, and the enzyme is inhibited by sodium or lithium ions.²² With certain yeast enzyme preparations, the addition of 30 to 100 micromoles of phosphate per milliliter increases the rate of the hydroxamate reaction considerably. The phosphate, however, is not indispensable, and the mechanism of its effect is not clear.

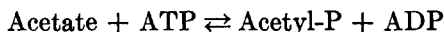
Effect of pH. The yeast enzyme is maximally active at pH 7.2 but has a rather broad optimum.⁶ The activity falls sharply as the pH is lowered below 7. Extracts of animal tissues appear to have a slightly more basic pH optimum, and for this reason they are assayed at pH 8.2 rather than 7.5.

ACTIVITY OF VARIOUS YEAST FRACTIONS

Fraction	Total volume, ml.	Units/ml.	Protein, mg./ml.	Specific activity, units/mg.	Total units	Recovery, %
1. Original extract (quick-frozen preparation)	1020	66	66	1.0	67,400	100
2. Protamine supernate	1000	66	33	2.0	66,000	98
3. Ammonium sulfate precipitate, 0-55%	132	495	97	5.1	65,200	97
4. Ammonium sulfate precipitate, 35-45%	32	1063	95	11.2	34,000	51

²² R. W. von Korff, *J. Biol. Chem.* **202**, 265 (1953).

[97] Acetate Kinase of Bacteria (Acetokinase)¹



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Assay Method

Principle. The reaction is carried out in the presence of excess hydroxylamine with acetate and ATP as reactants. The assay method makes use of the ability of acyl phosphates to form hydroxamic acids rapidly at neutrality and the subsequent formation of the colored ferric-hydroxamate complex in acid solution.²

¹ According to I. A. Rose, M. Grunberg-Manago, S. R. Korey, and S. Ochoa, to be published.

² F. Lipmann and L. C. Tuttle, *J. Biol. Chem.* **159**, 21 (1945).

Reagents

3.2 *M* potassium acetate.

1.0 *M* Tris-HCl buffer, pH 7.4.

1.0 *M* MgCl₂.

4.0 *M* KOH.

28% hydroxylamine hydrochloride. This reagent is stored in the cold.

0.1 *M* ATP. Sodium salt neutralized with HCl.

FeCl₃ reagent. 1.25% FeCl₃ in 1.0 *N* HCl.

10% trichloroacetic acid.

Phosphate-cysteine. 0.1 *M* potassium phosphate buffer, pH 7.4, containing 0.005 *M* cysteine.

Enzyme. Dilute stock enzyme with phosphate-cysteine to obtain 10 to 100 units/ml. (See definition below.)

Procedure. The stock of substrate is made up fresh daily using potassium acetate, Tris, and MgCl₂ in a volume ratio of 25:5:1. A neutral solution of hydroxylamine is made fresh by mixing equal volumes of the hydrochloride and KOH. Mix 0.3 ml. of the stock substrate, 0.35 ml. of neutral hydroxylamine, 0.1 ml. of ATP, and an amount of water to make the final volume with enzyme to 1.0 ml. Place the tube at 29°. Add one unit or less of enzyme at zero time, and stop the reaction after at least 2 minutes by the addition of 1.0 ml. of trichloroacetic acid. Centrifuge off any precipitate. Color is developed by adding 4.0 ml. of FeCl₃ reagent and is read immediately in the Klett colorimeter using the 540-m μ filter. A reading of 69 units above a reagent blank corresponds to 1.0 micromole of acetohydroxamic acid.

Definition of Unit and Specific Activity. One unit of enzyme is defined as the amount which produces 1 micromole of hydroxamic acid per minute under the standard assay conditions given above. The assay is linearly responsive to enzyme concentration below 2 units of enzyme per tube. Specific activity is defined as units per milligram of protein. Protein is determined by the biuret reaction.³ Alternatively, protein may be determined by the method of Warburg and Christian⁴ when the ratio of optical densities at 280 to 260 m μ is greater than 0.8.

Application of the Assay Method to Bacterial Extracts. Acetokinase has been found to be distributed widely among bacteria but has not been reported in mammalian tissues. The bacterial extracts are usually prepared by grinding with alumina and extracting by stirring with buffer

³ A. G. Gornall, C. J. Bardawill, and M. M. David, *J. Biol. Chem.* **177**, 751 (1949).

⁴ O. Warburg and W. Christian, *Biochem. Z.* **310**, 384 (1941-42).

(see below). Especially with crude extracts, a positive assay is not definite evidence for the presence of acetokinase in so far as the acetate-activating enzyme⁵ will give rise to acetohydroxamic acid in the presence of catalytic concentrations of CoA. The most rigorous method for ruling out this latter enzyme is by the inability of AMP plus pyrophosphate to replace ADP in causing the disappearance of acetyl phosphate in the presence of added transacetylase.⁵ An alternative procedure is to treat the enzyme solution with an anion exchange resin to remove CoA.

Purification Procedure from *E. coli*

Step 1. Preparation of Crude Extract. The starting material was a lyophilized powder of *E. coli*, strain 4157 (for preparation, see Vol. I [114]). This powder had been stored in the cold for two years. Six-gram amounts of an equal weight mixture of alumina and bacterial powder are ground at room temperature with wetting amounts of potassium phosphate buffer, 0.1 M, pH 7.4. The gummy paste is stirred with 5 vol. of buffer for 30 minutes at 2°. The accumulated mixture is centrifuged at 18,000 × *g* for 1 hour in the cold. The residue is extracted again with 2.5 vol. of buffer, stirred, and centrifuged as before, and the clear supernatant solutions are combined. The solution usually contains 10 to 14 mg. of protein per milliliter with a specific activity of 2 to 3. It may be stored with 0.005 M cysteine with no loss of activity.

Step 2. First Acetone Fractionation. The crude extract, pH 7.3, is brought to 45% concentration of acetone (by volume) beginning at 0° and ending at -10° with rapid stirring and dropwise addition of acetone (held at -10°). The heavy precipitate is removed by centrifugation at -10° for 1 hour at 500 × *g*. The clear supernatant fluid is then brought to 55% acetone concentration and again centrifuged. The carefully drained precipitate is well suspended at 0° in 0.1 vol. of 0.5 saturated⁶ ammonium sulfate containing 0.005 M cysteine. The enzyme assay may be carried out in the presence of ammonium sulfate in order to follow the progress of the purification. The enzyme cannot be stored at this point.

Step 3. First Ammonium Sulfate Fractionation. The above suspension is centrifuged in the cold to remove inactive protein; the supernatant should contain about 7 mg. of protein per milliliter. Solid ammonium sulfate is next added to the cold solution with stirring to obtain three fractions at 0.55, 0.60, and 0.65 ammonium sulfate saturation. The three precipitates are dissolved in small volumes of phosphate-cysteine and their specific activities determined. The best two fractions are combined. The

⁵ F. Lipmann, M. E. Jones, S. Black, and R. Flynn, *J. Am. Chem. Soc.* **74**, 2384 (1952).

⁶ Made up by adding 35.0 g. of ammonium sulfate to 100 ml. of water.

frozen solution may be stored indefinitely, and loss may be held to a minimum by the periodic addition of neutral cysteine.

Step 4. Second Acetone Fractionation. The solution from step 3 is diluted to contain 7 mg. of protein per milliliter and dialyzed for 2 hours against 10 vol. of phosphate-cysteine at 0°. Any insoluble material is removed, and the solution is fractionated as before with acetone at -10°. Three fractions are collected between 45 and 50, 50 and 55, and 55 and 60% acetone concentration. The major part of the activity usually appears in the 50 to 55% fraction. The precipitates, taken up in a little phosphate-cysteine, cannot be stored.

Steps 5 and 6. Second and Third Ammonium Sulfate Steps. The most active fraction of the previous step is diluted to 5.0 mg. of protein per milliliter and adjusted to pH 6.7. The protein precipitating between 0.0 and 0.45 ammonium sulfate saturation is discarded. Three further fractions are obtained between 0.45 and 0.50, 0.50 and 0.55, and 0.55 and 0.60% ammonium sulfate saturation, and the most active combined as before.

Another 1.5-fold purification is achieved by repeating this step in successive 0.02 saturation fractions between the limits of 0.45 and 0.55 ammonium sulfate saturation. The highest specific activity achieved was about 300. The preparation is free of adenylic kinase (myokinase) and transacetylase. It is stored in phosphate-cysteine at -20°.

Properties

Specificity. Of a number of acids tested, acetate and propionate are the only ones which serve as substrate, acetate being ten times as active as propionate. No measurable reaction is obtained with fluoracetate. ADP cannot replace ATP as the primary phosphorylating agent. The following Michaelis constants were found: acetate, 0.3 *M*; propionate, 0.47 *M*; Mg⁺⁺, 0.005 *M*; ATP, 0.002 *M*; ADP, 0.0015 *M*; acetyl phosphate, 0.005 *M*. The V_{max} in the back direction is five times that in the forward direction. (The rate in the back direction is determined by the disappearance of acetyl phosphate, the reaction being stopped with *p*-chloromercuribenzoate before the addition of hydroxylamine.) The apparent equilibrium constant at pH 7.0 is 0.006. Thus the equilibrium favors the formation of ATP from acetyl phosphate + ADP.

Activators and Inhibitors. Mg⁺⁺ or Mn⁺⁺ is required for the reaction. High concentrations of Versene may be used to stop the reaction.

The following sulfhydryl reagents produce 50% inhibition of the reaction: Hg⁺⁺, 10⁻⁷ *M*; *p*-chloromercuribenzoate, 3 × 10⁻⁷ *M*; iodosobenzoate, 3 × 10⁻⁴ *M*; and phenylarsine oxide, 10⁻⁸ *M*. The inhibitions

caused by the first three compounds are largely reversed by cysteine. Iodoacetate and iodoacetamide do not inhibit at 0.01 *M*. Fluoroacetate is not an inhibitor.

Effect of pH. The enzyme activity is optimum at pH 7.4 in either direction, the rate falling moderately on both sides. Its stability is limited to the range 6.5 to 7.8.

Distribution. Assaying extracts from alumina ground cells, the following organisms are found to give solutions with the noted specific activities of acetokinase: *Proteus vulgaris*, 10.8; *Clostridium* strain H.F., 5.9; *E. coli*, 3.0; *Streptococcus faecalis*, 2.7; *Clostridium kluverii*, 0.04; *Streptococcus haemolyticus*, 0.4; and *Azotobacter vinelandii*, 0.05.

Determination of Acetate with Acetokinase. Acetokinase may be used readily for the quantitative determination of micromole amounts of acetate. To a tube containing ATP (0.01 *M*), MgCl₂ (0.01 *M*), Tris (0.05 *M*), neutral hydroxylamine (0.75 *M*) and 6 to 12 units of acetokinase from *E. coli*, add an amount of the neutralized solution to be tested which would contain 0.5 to 2.5 micromoles of acetate and adjust the volume to 1.0 ml. Incubate at 29 to 38° for 1 hour, and terminate the reaction with 1.0 ml. of 10% TCA. Color is developed with 4.0 ml. of FeCl₃ reagent and read in the colorimeter as above. This method has been applied with better than 95% recoveries to crude biological mixtures. Acetokinase prepared through *step 3* of the purification procedure is satisfactory for use in the determination.

SUMMARY OF PURIFICATION PROCEDURE

Fraction	Total volume, ml.	Total units	Total protein, mg.	Specific activity	Recovery, %
1. Extract ^a	410	12,200	4060	3.1	100
2. Acetone, 45-55%	56	6,750	450	15	55
3. AmSO ₄ , 0.5-0.6	21	6,100	140	42	49
4. Acetone, 50-55%	6	2,900	30	98	24
5. AmSO ₄ , 0.50-0.6	3	2,400	13	190	20
6. AmSO ₄ , 0.49-0.53	2	1,800	6	300	15

^a Extract from 36 g. of dry cells.