Formate

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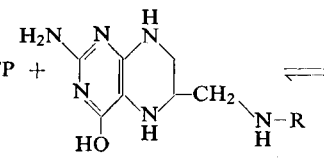
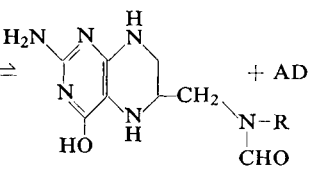
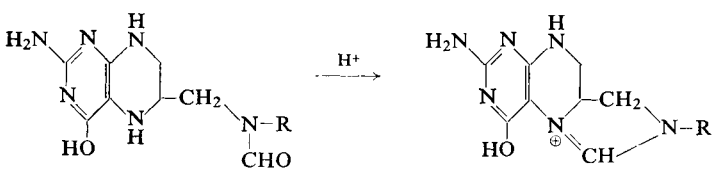
Principle

Tetrahydrofolic acid formylase catalyses the formylation of tetrahydrofolic acid (THF) in the presence of adenosine triphosphate (ATP)[1] to give N(10)-formyl-tetrahydrofolic acid (I):

(1) Formate + ATP +  ⇌  + ADP + phosphate

THF (I)

The reaction product (I) is converted to 5,10-methenyl-tetrahydrofolic acid (II) in the presence of acid[2,3]:

(2) 

(I) (II)

In the presence of an excess of THF and ATP reaction (1) proceeds quantitatively from left to right[4]. Product (II) has an absorption maximum at 350 mμ and therefore formate can be determined by measuring the increase in optical density at this wavelength.

Reagents

1. Triethanolamine
   freshly distilled, b. p. 277—279° C/150 mm.
2. Adenosine triphosphate, ATP
   sodium salt, ATP-$Na_2H_2 \cdot 3\,H_2O$; commercial preparation, see p. 1006.
3. Magnesium chloride, $MgCl_2 \cdot 6H_2O$
4. Sodium formate
5. Hydrochloric acid, A. R., conc.
6. Potassium hydroxide, A. R., 2 N
7. 2-Mercaptoethanol
8. Perchloric acid, sp. gr. 1.67, *ca.* 70% (w/w)
9. DL-Tetrahydrofolic acid, THF
   as the acetate, prepared according to[4] from folic acid (commercial preparation, see p. 1013). For a description of the preparation, see the Appendix, p. 311.

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1) *G. R. Greenberg, L. Jaenicke* and *M. Silverman*, Biochim. biophysica Acta *17*, 589 [1955].
2) *M. May, T. J. Bardos, F. L. Barger, M. Lansford, J. M. Ravel, G. L. Sutherland* and *W. Shive*, J. Amer. chem. Soc. *73*, 3067 [1951].
3) *D. B. Cosulich, B. Roth, J. M. Smith jr., M. E. Hultquist* and *R. P. Parker*, J. Amer. chem. Soc. *74*, 3252 [1952].
4) *J. C. Rabinowitz* and *W. E. Pricer jr.*, J. biol. Chemistry *229*, 321 [1957].

10. Tetrahydrofolic acid formylase
obtained from *Clostridium cylindrosporum* according to[4,5]. For a description of the preparation, see Appendix, p. 312.

Purity of the enzyme preparations
The relatively crude preparation with a specific activity of about 900 units*)/mg. described on p. 312 is suitable. It contains adenylic acid kinase, so that the assay in the form described here is not suitable for the determination of ATP. The enzyme has also been obtained in a crystalline state with a specific activity of 35000 units/mg.[6,7]. The crystalline enzyme is free from adenylic acid kinase, ATPase or nucleoside diphosphokinase and is suitable for the determination of ATP, THF or formate.

Preparation of Solutions

I. Triethanolamine buffer (1.0 M; pH 8.0):
Dissolve 149 g. triethanolamine in 750 ml. distilled water, adjust to pH 8.0 with conc. HCl and dilute to 1000 ml. with distilled water.

II. DL-Tetrahydrofolic acid (*ca.* 0.01 M THF; pH 7.0):
Dissolve 56 mg. of the acetate prepared according to p. 311 in 10 ml. 1 M 2-mercaptoethanol (dilute 7 ml. mercaptoethanol to 100 ml. with distilled water) and adjust the pH to 7.0 with 2 N KOH (7 to 10 drops).

III. Adenosine triphosphate (0.05 M ATP; pH 7.0):
Dissolve 151.3 mg. ATP-$Na_2H_2 \cdot 3H_2O$ in 4 ml. distilled water, adjust to pH 7.0 with 1 N KOH and dilute to 5 ml. with distilled water.

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

V. Sodium formate standard solution (10^{-3} M; pH 7.0):
Dissolve 68 mg. sodium formate in 50 ml. distilled water, adjust to pH 7.0 with 1 N KOH and dilute to 100 ml. with distilled water.

VI. Perchloric acid (*ca.* 2% w/v):
Dilute 2 ml. 70% $HClO_4$ to 120 ml. with distilled water.

VII. Tetrahydrofolic acid formylase (*ca.* 9000 units/ml.):
Dissolve 20—30 mg. of the dry powder obtained according to p. 312 in 2 ml. 0.01 M 2-mercaptoethanol (dilute 0.7 ml. mercaptoethanol to 1000 ml. with distilled water).

Stability of the solutions
Store the ATP solution at −20° C. Keep all other solutions, including the enzyme solution, at 0—4° C. Store the THF solution in evacuated and sealed glass tubes. It is stable for 2 to 4 weeks.

Procedure

Experimental material
Neutralize urine samples. They can be analysed without further treatment. Formic acid in blood is separated by lyophilization of a sample acidified with H_2SO_4 and then the neutralized

*) A unit is the amount of enzyme which, under the conditions described here, changes the optical density at 350 mμ by 1.0 in 10 min. This corresponds to the formation of 0.012 μmoles N(10)-formyl-THF/min.

5) *J. C. Rabinowitz* and *H. A. Barker*, J. biol. Chemistry *218*, 161 [1956].
6) *J. C. Rabinowitz* and *W. E. Pricer jr.*, Fed. Proc. *17*, 293 [1958].
7) *J. C. Rabinowitz* and *R. H. Himes*, Fed. Proc. *19*, 963 [1960].

distillate is used for the assay. Formate formed in the periodate oxidation of polyhydroxy compounds can also be determined. Concentrations of $NaIO_3$ up to 5 μmoles/ml. and $NaIO_4$ up to 3.6 μmoles/ml. do not interfere with the assay[8].

Enzymatic reaction

Wavelength: 350 mμ; light path: 1 cm.; final volume: 3.0 ml. Measure against the control.

Prepare a mixture of the following solutions:

1 vol. buffer (solution I)
1 vol. ATP solution (III)
1 vol. $MgCl_2$ solution (IV)
2 vol. THF solution (II).

Pipette into test tubes (1.1 cm. diameter, 10 cm. long):

	Experimental	*Blank 1*	*Blank 2*	*Control*
reagent mixture	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
sample*) or standard soln. (V)	0.4 ml.	0.4 ml.	—	—
distilled water	—	0.1 ml.	0.4 ml.	0.5 ml.

Place all the tubes in a water bath at 37°C for 2 min. Pipette into the experimental and blank 2 tubes

0.1 ml. enzyme solution (VII) (= 500 to 1000 units)

with a Lang-Levy micropipette**). Wait for 30 sec. between each addition of the enzyme. Incubate each tube for exactly 10 min. at 37°C (calculated from the time of the enzyme addition). Then pipette into all tubes

2.0 ml. perchloric acid (solution VI).

Centrifuge off the precipitated protein and 10 to 30 min. after the addition of the acid measure the optical densities of the experimental tube (E_E) and the two blanks (E_1, E_2) against the control tube.

Calculations

The extinction coefficient of 5,10-methenyl-tetrahydrofolic acid at 350 mμ is 24.9 cm.²/μmole. Therefore it follows:

$$\frac{\Delta E \times 3}{24.9 \times V_S} = \frac{\Delta E}{8.3 \times V_S} = \text{μmoles formate/ml. sample}$$

where

$\Delta E = E_E - E_1 - E_2$
E_E = optical density of the experimental tube
E_1 = optical density of the blank 1
E_2 = optical density of the blank 2
3 = final volume [ml.]
V_S = volume of sample taken for the assay [ml.]

*) Containing 0.01 to 0.1 μmoles formate.
**) Obtainable from H. E. Pedersen, Copenhagen, Denmark.
8) *J. C. Rabinowitz* and *D. H. Rammler*, Anal. Biochem. *4*, 116 [1962].

Example

A urine sample was analysed. The volume of sample taken for the assay was 0.1 ml. $E_E = 0.337$; E_1 (no enzyme) = 0.066; E_2 (no urine) = 0.097. $\Delta E = 0.337 - 0.066 - 0.097 = 0.174$.

$$\frac{0.174}{8.3 \times 0.1} = 0.21 \text{ μmoles formate/ml. urine}$$

Sources of Error

The optical density of blank 2 may be relatively high if the THF solution contains decomposition products. If the optical density (measured against the control) is higher than 0.2, prepare a fresh THF solution.

Specificity

The following compounds did not react (in concentrations of 0.01 to 0.1 μmoles/assay mixture): methanol, formaldehyde, formamide, acetate, pyruvate, formamidine, glycine, formylglycine, formylaspartate, formylglutamate, formylanthranilate, formiminoaspartate, formiminoglutamate, DL-serine, xanthine, inosinic acid, potassium phosphite. 0.1 μmoles Formiminoglycine reacted slightly, probably due to its enzymic conversion to glycine, formate and ammonia.

Other Methods for the Determination of Formate

The most sensitive is the reduction of formate to formaldehyde, and determination of the latter with chromotropic acid[9]. The reduction and the colour formation require strongly acid conditions. This method is therefore not suitable for the determination of formate in the presence of acid-labile formyl derivatives, which often occur in biological material. Other methods depend on the manometric determination of the CO_2 or H_2 formed from formic acid by enzyme preparations from *Escherichia coli*[10, 11], by lead tetraacetate[12], or by ceric sulphate[13]. They have the disadvantage of requiring relatively large amounts of formate (2.5 to 20 μmoles/ml. sample), of employing enzyme preparations which are only stable for 24 hours[10], of reacting with pyruvic acid[11] or of requiring preliminary separation of the formic acid by steam distillation[10, 13].

Appendix

Preparation of tetrahydrofolic acid (THF)

Folic acid is reduced to tetrahydrofolic acid with H_2 in the presence of a platinum oxide catalyst at atmospheric pressure[4]. Suspend 100 mg. platinum oxide in 25 ml. acetic acid, reduce with H_2 and then add 500 mg. folic acid in 25 ml. acetic acid from the side-arm of the reaction vessel. Stir magnetically until the H_2-uptake is complete. Filter the solution of tetrahydrofolic acid through a coarse, sintered glass funnel packed with a celite pad which has been previously washed with acetic acid. Collect the filtrate in a vessel which can be connected to a lyophilization apparatus. Lyophilize the filtrate in the dark. Store the residue (DL-tetrahydrofolic acid acetate, molecular weight: 560; white powder) in an evacuated desiccator at −10°C. Under these conditions the material slowly turns brown. However, a preparation which had lost 40 to 50% of its activity was still satisfactory in the assay.

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9) *W. M. Grant*, Analytic. Chem. *20*, 267 [1948].
10) *R. E. Asnis* and *M. C. Glick*, J. biol. Chemistry *220*, 691 [1956].
11) *H. Gest* in *W. D. McElroy* and *B. Glass:* Phosphorus Metabolism. The Johns Hopkins Press, Baltimore 1952, Vol. 2.
12) *A. S. Perlin*, Analytic. Chem. *26*, 1053 [1954].
13) *M. J. Pickett*, *H. L. Ley* and *N. S. Zygmuntowicz*, J. biol. Chemistry *156*, 303 [1944].

Preparation of tetrahydrofolic acid formylase[4])

Suspend 2 g. lyophilized cells[5, 14] of *Clostridium cylindrosporum* in 40 ml. 0.05 M potassium maleate buffer (pH 7.0; 0.05 M with respect to 2-mercaptoethanol), allow to stand for 30 min. at 37° C and then centrifuge for 10 min. at 40000 r.p.m. (105000 g) (rotor No. 40 of the Spinco ultracentrifuge). Pour 8 ml. of the supernatant into each of 4 centrifuge tubes, containing 0.01 ml. mercaptoethanol, cool to −8° C (alcohol-ice) and add 5.6 ml. of cold acetone to each tube (0.1 M with respect to 2-mercaptoethanol). Centrifuge at −10° C in the Servall centrifuge (30000 g). Discard the precipitates. To the supernatant from each tube add 2.2 ml. acetone (0.1 M with respect to 2-mercaptoethanol), centrifuge at −10° C and dissolve the combined precipitates in 4 ml. 0.02 M potassium maleate buffer (0.01 M respect to 2-mercaptoethanol). The solution contains about 9000 units/ml. (10 to 15 mg. protein/ml.). It should be stored in an evacuated and sealed container at 2° C, and it keeps for 2 to 4 weeks in this state. The solution can also be lyophilized and the dry powder may be stored *in vacuo* at −10° C for at least 6 weeks.

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[14]) *J. C. Rabinowitz* in *P. D. Boyer, H. A. Lardy* and *K. Myrbäck:* The Enzymes. 2nd ed., Academic Press, New York 1960, Vol. 2.