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Study of 3-Ketosteroid Dehydrogenase System Using Whole-cell-enzyme from *Arthrobacter simplex*

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Abstract—A new assay method for delta-1-dehydrogenated-3-ketocorticosteroid in the presence of proteinous material or whole-cell-enzyme and 3-ketocorticosteroid has been developed. This method makes use of the linear relationship between the ratio of absorbances at 265 nm and at 242 nm and the fractional concentration of delta-1-3-ketosteroid. Theoretical values were calculated based on the absorbances of proteinous material at fixed concentrations of the 3-ketosteroid and delta-1-dehydrogenated-3-ketosteroid. The values obtained experimentally showed good agreement with the values theoretically predicted. The new assay method developed for the steroid mixture containing proteinous material is of some practical importance. The use of such assay method enables one to determine the enzyme activity and the rate of enzyme reaction or conversion rather quickly, easily and accurately. By the use of this assay method, the reaction kinetics of whole-cell-enzyme has also been studied. It was found that it followed the simple Michaelis-Menten type enzyme kinetics. Also the reversibility of this reaction with actively metabolizing cell was examined. It was found that delta-1-dehydrogenated-3-ketosteroid could not be hydrogenated reversibly to 3-ketosteroid by this enzyme system.

In the case of 3-ketosteroid-delta-1-dehydrogenase reaction system, the dehydrogenated product shows almost the same chemical and physical properties as substrate. These similar properties of the substrate and product make it difficult to assay them quantitatively. In literature, several assay methods for steroids product were reported, but the product had to be separated from the mixture prior to the assay. Chromatography[1], polarographic method [2,3], and colorimetric method[4-8] are some of these examples. Chromatographic method have limited application for the enzyme assay since it takes too much time and also it is not so accurate as one might like. Colorimetric methods are based on differential rate of color formation where relatively small amount of one compound exists in the presence of relatively large amount of other compounds. For this reason, this method can not usually be employed for enzyme kinetic studies. In this paper, we are going to report a new, accurate, and convenient assay method for this dehydrogenase system and the reaction kinetics of whole-cell-enzyme.

EXPERIMENTAL

Microorganism—*Arthrobacter simplex* (ATCC 6946) on nutrient agar slant stored in cold room maintained at 0~5° was used for production of 3-ketosteroid-delta-1-dehydrogenase throughout this study. The slant cultures were transferred to fresh slant once every 4 weeks.

Enzyme assay—One ml of reaction mixture was extracted with 2 ml of ethylacetate. Ethylacetate was evaporated at room temperature. The once crystalized solid material was redissolved in proper amount of absolute ethanol to give the UV absorption at 242 nm in the range of 0.7~0.9. The fractional conversion was calculated according to the standard curve.

Enzyme induction—The first stage inoculum was prepared in the same medium and by the same method as was used by Ryu *et al*[9]. 5% of first stage inoculum was used as the seed culture for enzyme production stage culture. 100 ml of enzyme production medium contains 4 g of Casitone Bacto(Difco), 0.1 g of K_2HPO_3 and 0.5 g of glucose. Culture was grown on reciprocating shakers at constant temperature, 30°. Progesterone in dimethylformamide (DMF) was used as enzyme inducer. 200 mg of progesterone dissolved in 1 ml of DMF was added to 1-liter of broth at 16 hr. after inoculation.

Whole-cell-enzyme-preparation—3-ketosteroid-delta-1-dehydrogenase induced whole cell was harvested by centrifugation at 3000 rpm for 15 min., followed by washing twice with the same aliquot of 0.01 M phosphate buffer(pH 7) as the broth. Whole cell was resuspended in acetone. The acetone was removed by centrifugation at 3000 rpm for 15 min. The remaining acetone was removed by freeze drying under vacuum, followed by heat treatment at 60° for 10 min. The dried whole-cell-enzyme could be stored at 0~5° refrigerater without loss of activity for 2 months.

Kinetic study—0.5 g of whole-cell-enzyme was reacted with hydrocortisone in the presence of external hydrogen acceptor, menadione. The reaction was carried out in 10 ml of phosphate buffer solution (pH 7, 0.01 M) in 30° water bath with occasional shaking. 0.43 mM of menadione was used for 1 M of substrate, hydrocortisone. The fractional conversion of hydrocortisine to prednisolone was measured at 5, 10, 20, 30, 60, and 120 minutes after addition of substrate by the same method as described in the enzyme assay. The reaction mixture without substrate was used as the blank.

Reaction with metabolizing cells—The dehydrogenase was induced by the addition of prednisolone at the end of logarithmic growth phase. The conditions used for enzyme induction was the same as that for whole-cell-enzyme induction. Reaction was carried out in 500-ml Erlenmeyer flask. 5 ml of reaction mixture was taken out at 1.0, 2.0, 2.5, 3.5, 4.5, and 7.5 hours after the inducer addition. Fractional conversion was calculated according to standard curve. The aliquot of non-fermented broth was used as blank instead of reaction mixture for enzyme assay. For the forward reaction, hydrocortisone was used as enzyme inducer in the same system as for the prednisolone. Fractional conversion was measured at 1.0, 2.0, 2.5, 3.5, 4.5, and 7.5 hours after inducer addition.

RESULTS AND DISCUSSION

UV absorbancy of hydrocortisone and prednisolone is shown in Fig. 1. As shown in Fig. 1, the maximum difference UV absorption appears at 265 nm. Similar results have been

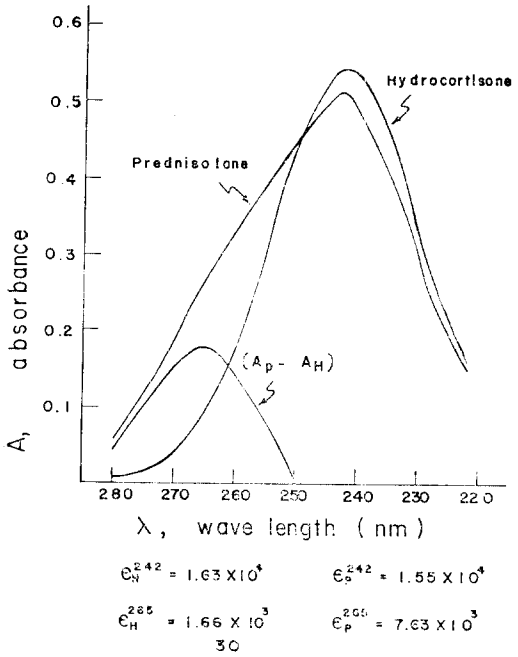

Fig. 1—UV Absorption spectrum for hydrocortisone and prednisolone.
(a) Hydrocortisone (b) Prednisolone
(c) Prednisolone-Hydrocortisone

reported by Ivashkiv[10]. When the atio of absorbance at 242 nm to that at 265 nm was plotted against fractional conversion, linear relationship was obtained as shown in Fig. 2. This correlation was found to be independent of absolute amount of total steroid and shows good agreement with theoretical values. We could not use this linear relationship for enzyme assay because of interference effect due to solvent extractable proteinous material. Ivashkiv[10] made use of linear relationship for assay of both the substrate and product by selective extraction of 16-hydroxycorticosteroids with borate buffer. In the case of hydrocortisone and prednisolone, we could not

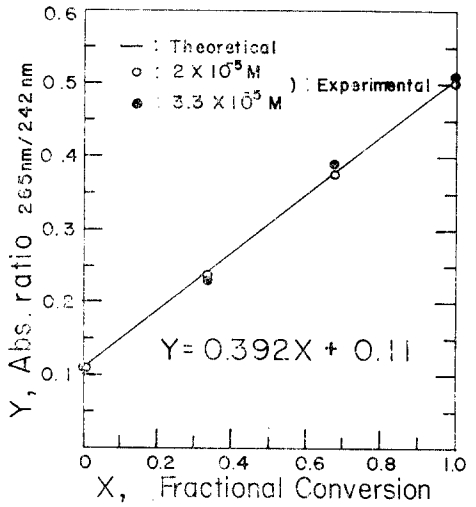

Fig. 2—Calibration for determination of steroid mixture.
— : Theoretical
○ : Total steroid: 2×10^{-5}M
● : Total steroid: 3.3×10^{-5}M

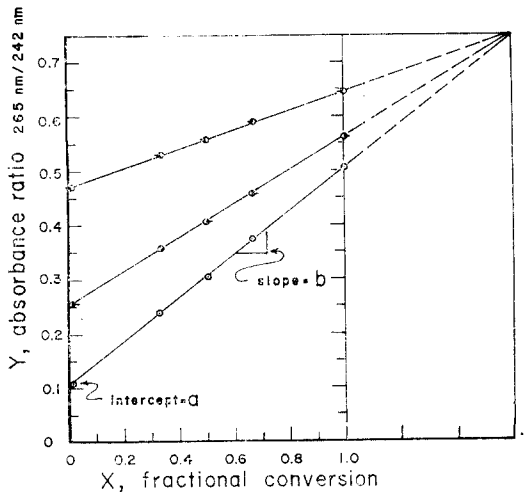

Fig. 3—Effect of whole-cell-enzyme extract on calibration for steroids.
◇ = 2.49×10^{-5}M, with whole-cell-enzyme
◆ = 1.10×10^{-4}M, with whole-cell-enzyme
⊙ = without whole-cell-enzyme

use borate salt, and we had to overcome this problem by modified method. For this reason, correction of the calibration curve (Fig. 3) was necessary. Theoretically, the absorbance ratio (265 nm to 242 nm) can be expressed as follows.

$$\text{Abs. ratio}=\frac{H265+P265+M265+C265}{H242+P242+M242+C242}$$

where H265: U.V. absorbance of hydrocortisone at 265 nm
=(conc. of hydrocortisone)×(molar extinction coeff. of hydrocortisone at 265 nm)

H242: UV absorbance of hydrocortisone at 242 nm

M265: UV absorbance of manadione at 265 nm

M242: UV absorbance of menadione at 242 nm

P265: UV absorbance of predinisolone at 265 nm

P242: UV absorbance of predinisolone at 242 nm

C265: UV absorbance of solvent extractable cellular material at 265 nm

C242: UV absorbance of solvent extractable cellular meterial at 242 nm

The effect of menadione on UV absorbance was negligible because of its small molar ratio to substrate and/or product. Therefore, absorbance ratio at two wave lengths could be calculated by using the molar extinction coefficient of hydrocortisone and predinisolone, their concentration and the value obtained from blank absorbance. At fixed concentration of whole-cell-enzyme, we found that the increase in substrate concentration decreased the slope and increased the intercept of the standard correlation curve. This assay method is very accurate, rapid, simple and reproducible. Thus this method could be applied to other similar systems.

Kinetic study—The whole-cell-enzyme kinetics was investigated in the substrate concentration range of 0.8 mM to 5 mM. The results showed that the whole-cell-enzyme exhibited simple Michaelis-Menten type enzyme kinetics (Fig. 4). The value of Km and the maximum reaction velocity determined are 1.57×10^{-3}M, 2.07×10^{-4} mole/min, respectively.

Conversion with metabolizing cells—The reversibility of dehydrogenase reaction with

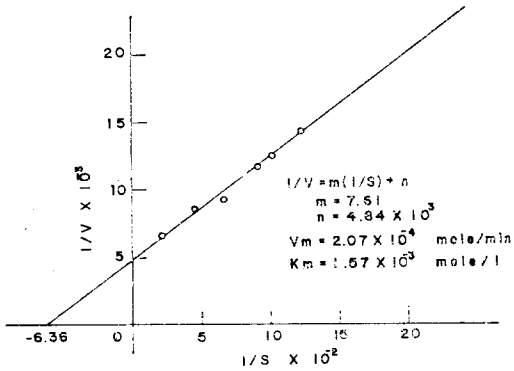

Fig. 4—Lineweaver-Burk plot for 3-ketosteroid-delta-1-dehydrogenase(whole-cell-enzyme)

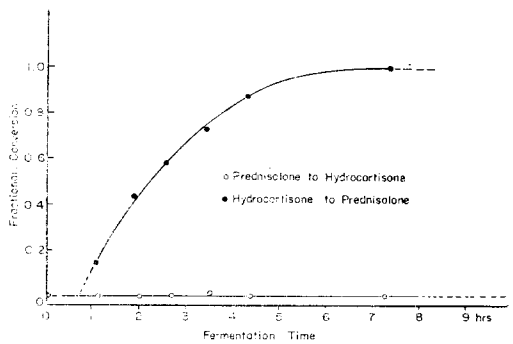

Fig. 5—Fractional conversion of hydrocortisone to prednisolone and prednisolone to hydrocortisone by actively metabolizing *A. simplex*.

actively metabolizing cells was examined. The result is shown at Fig. 5. With the metabolizing cells, hydrocortisone could be dehydrogenated to prednisolone irreversibly. Our result was different from that of Penasse *et al.*[11] in that they found the reversibility of the reaction with purified enzyme in anaerobiosis. Aeration of metabolizing cell may affect the reversibility of dehydrogenase. We need further study of this aeration effect on dehydrogenase.

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