In vitro Conversion of Sepiapterin to Isosepiapterin via Dihydrobiopterin

The mutant *sepia* of *Drosophila melanogaster* is characterized by accumulation of 2 yellow dihydropteridines, sepiapterin and isosepiapterin, in the eye. In spite of their structural similarities[1], the metabolic or biochemical relationship between these pteridines has not been elucidated.

In a recent paper, we have described evidence showing that sepiapterin is reduced in the presence of reduced nicotinamide dinucleotide phosphate (NADPH) to dihydrobiopterin by a sepiapterin reductase from rat liver[2]. During the course of these studies, we noticed that if the reaction mixture of sepiapterin and the enzyme was treated with diluted acids after almost complete reduction of the pteridine, a strong blue fluorescent substance was produced together with a small amount of yellow fluorescent material which was distinguishable from sepiapterin by paper chromatography. The blue fluorescent compound was identified as biopterin by its characteristic UV-absorption spectrum and paper chromatographic behaviour[2,3]. By paper chromatographic analysis, we postulated that the minor yellow compound was identical with isosepiapterin and was spontaneously derived from dihydrobiopterin by acid treatment; now we present evidence in favour of this inference.

To isolate dihydrobiopterin from the reaction mixture of sepiapterin and its reductase, we adopted essentially the methods described by MATSUBARA[3]. The reaction mixture, in a final volume of 26.6 ml, was incubated at 37 °C for 35 min in the dark, and contained: potassium phosphate buffer, pH 6.8, 200 mM; sepiapterin[4], 9.2 μmoles; NADPH (prepared from NADP by the method of ZAKRZEWSKI[5]), 8.0 μmoles; glucose-6-phosphate dehydrogenase[6] (742 μg protein/ml), 2.0 ml; and sepiapterin reductase from rat liver[2] (916 μg protein/ml), 8.0 ml. The almost colourless reaction mixture was applied to a Sephadex G-50 column (3 · 22 cm) that was eluted with distilled water. Dihydrobiopterin fractions were combined and lyophilized. The yield was approximately 8.0 μmoles. The concentration of dihydrobiopterin was calculated from optical density at 330 nm[3]. Protein was determined by the method of LOWRY et al.[7].

The lyophilized powder was dissolved in 1.5 ml of 0.5 N sulphuric acid and stored overnight in a deep freezer (− 20 °C). The thawed solution was neutralized with a saturated solution of barium hydroxide. After centrifugation, the yellow fluorescent supernatant solution was applied to a Sephadex G-50 column (1 · 22 cm) that was eluted with distilled water. The combined yellow eluate was subjected to the following analyses. The yield was about 0.85 μmoles (as isosepiapterin). The concentration of isosepiapterin was estimated from the extinction coefficient at 430 nm in 0.1 N NaOH.

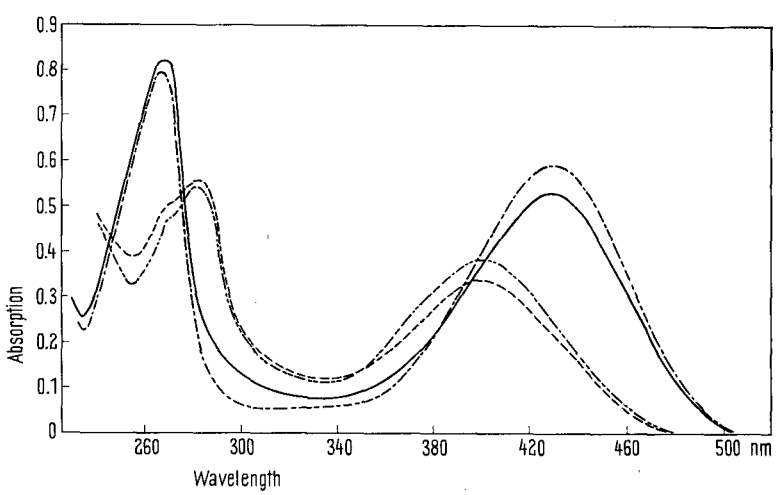
Paper chromatographic behaviour of the yellow compound is identical with that of isosepiapterin obtained from *sepia* flies[4] (Table). Its UV-absorption spectrum is practically the same as that of isosepiapterin (Figure).

Comparison of Rf values of the yellow compound and isosepiapterin

Solvent system	Rf value: Yellow compound	Rf value: Isosepiapterin
1	0.49	0.50
2	0.44	0.44
3	0.57	0.57
4	0.61	0.61
5	0.65	0.65
6	0.69	0.69
7	0.24	0.23
8	0.23	0.22

Solvent system: (1) *n*-propanol-ethylacetate-water (7:1:2, v/v); (2) 95% ethanol-*n*-amylalcohol-water (7:5:3); (3) *n*-butanol-acetic acid-water (4:1:2); (4) *iso*-propanol-water (7:3); (5) *n*-propanol-1% ammonia (2:1); (6) *n*-propanol-1% ammonium acetate (1:1); (7) 3% ammonium chloride; (8) 4% sodium citrate. Ascending method. Toyo Filter Paper, No. 50.

[1] H. S. FORREST and S. NAWA, Nature *196*, 372 (1962).
[2] M. MATSUBARA, S. KATOH, M. AKINO, and S. KAUFMAN, Biochim. biophys. Acta, in press.
[3] M. MATSUBARA, in preparation.
[4] M. TSUSUE and M. AKINO, Zool. Mag., Tokyo *74*, 91 (1965).
[5] S. F. ZAKRZEWSKI, J. biol. Chem. *235*, 1776 (1960).
[6] A. KORNBERG and B. L. HORECKER, in *Methods in Enzymology* (Ed. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York 1955), vol. 1, p. 323.
[7] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).



Absorption spectra of the yellow compound and isosepiapterin. The yellow compound: —— = in 0.1 N NaOH; ---- = in 0.1 N HCl. Isosepiapterin: —·—· = in 0.1 N NaOH; —·—· = in 0.1 N HCl.

The absorption maximum of fluorescence spectrum (515 nm at neutral pH) was also coincident with that of isosepiapterin. Furthermore, on alkaline permanganate oxidation the compound gave 2-amino-4-hydroxypteridine-6-carboxylic acid (Rf = 0.03, solvent system 1 in the Table). Hydrogen peroxide oxidation gave both xanthopterin (Rf = 0.10, solvent system 1) and 2-amino-4-hydroxypteridine-6-carboxylic acid. In alkaline solution, the compound was readily oxidized with bromine to xanthopterin. Labile hydrogen was determined by iodine titration[8,9]. Iodine equivalent to 0.81 μmoles of hydrogen was consumed per μmole of the compound. The carbonyl group of the compound was determined by the 2,4-dinitro-phenylhydrazine method[10] and gave 1.07 μmoles of carbonyl group per μmole. These chemical characteristics are consistent with those of isosepiapterin described by FORREST et al.[11,12]. From the above results it can be concluded that the yellow compound is identical with isosepiapterin. Besides acid treatment, adsorption of dihydrobiopterin at neutral pH on Filtrol Grade 58 followed by elution with 20% aqueous acetone also leads to the formation of isosepiapterin. Biopterin gives no oxidized form of isosepiapterin by similar treatment. Studies on the biological significance of the reaction are being undertaken.

Résumé. La dihydrobioptérine obtenue par le mélange réactionel de sépiaptérine et de sa réductase se transforme en substance jaune par traitement à l'acide sulfurique. Employant des techniques chromatographiques, l'analyse du spectre d'absorption UV et l'analyse chimique, on a pu mettre en évidence que cette substance est identique de l'isosépiaptérine.

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Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo (Japan), July 4, 1966.

[8] I. M. KOLTHOFF and M. N. SANDELL, *Textbook of Quantitative Inorganic Analysis* (Macmillan Co. Ltd., New York 1952), 3rd ed., p. 585.
[9] T. SHIOTA and M. N. DISRALEY, Biochim. biophys. Acta *52*, 467 (1961).
[10] H. M. KALCKAR, N. O. KJELDGAARD, and H. KLENOW, Biochim. biophys. Acta *5*, 586 (1950).
[11] H. S. FORREST, C. VAN BAALEN, and J. MYERS, Arch. Biochem. Biophys. *83*, 508 (1959).
[12] H. S. FORREST, D. HATFIELD, and C. VAN BAALEN, Nature *183*, 1269 (1959).

Changes in Alanine Transaminase Activity in the Liver of Riboflavin-Deficient Rats

NICHOL et al.[1] and EISENSTEIN[2] observed a direct correlation between alanine transaminase activity and glycogen deposition in liver through gluconeogenesis. They have studied this association in various ways, including the use of pyridoxine-deficient rats. It has also been reported that alanine transaminase activity varies according to the size of the free amino acid pool in the liver[1].

In riboflavin deficiency MORGAN et al.[3,4] observed an increased gluconeogenesis in the liver at the beginning of riboflavin deficiency. After 5 weeks on riboflavin-deficient diet, rats showed depressed gluconeogenetic activity. These workers subjected the normal and deficient rats to physiological stress and observed an impaired gluconeogenetic response to stress in riboflavin deficiency, which was attributed to the failure of pituitary adrenal axis or 'trigger mechanism' to release ACTH by the pituitary.

When normal rats were injected with deoxycorticosterone, hepatic alanine transaminase activity was lowered. This inhibitory effect of deoxycorticosterone on alanine transaminase activity was suggested to be due to the suppression of ACTH release by the pituitary[1]. This situation is similar to that observed in riboflavin-deficient rats by MORGAN et al.[4].

If such a defect exists under riboflavin deficiency, it is natural to expect low activity of alanine transaminase in the liver of riboflavin-deficient rats. On the contrary, it has been reported that there is increased activity of this enzyme in the liver of riboflavin-deficient rats[5].

In the present investigation, changes in alanine transaminase activity, glycogen and pyridoxal phosphate concentration of liver have been studied in riboflavin deficiency.

Young male albino rats of 80–100 g were divided into 2 groups of equal average body weights. Group A consisted of normal animals, and Group B of riboflavin-deficient rats. The animals were pair-fed on 16% protein for 45 days. Particulars regarding the diet have been reported elsewhere[6]. Water-soluble vitamins were supplied daily by subcutaneous injection.

After the experimental period was over, the rats were kept fasting for 24 h and then decapitated. The livers were removed, cleaned of adherent blood, weighed, and chilled in ice. A weighed quantity of liver was digested in 30% KOH and used for the determination of glycogen[7]. Another part of the liver was homogenized in phosphate buffer of pH 7.4 and used for the determination of alanine transaminase activity[8] and pyridoxal phosphate concentration[9]. It can be seen from the accompanying Table that alanine transaminase activity and the concentration of pyridoxal phosphate and glycogen in liver are increased in riboflavin deficiency.

In riboflavin deficiency, the oxidative enzyme system is affected by decreased concentration of flavin enzymes[10]. Therefore the oxidation of amino acid is ex-

[1] C. A. NICHOL and F. ROSEN, in *Advances in Enzyme Regulation* (Ed. G. WEBER; Pergamon Press, London 1964), vol. I, p. 341.
[2] A. B. EISENSTEIN, Endocrinology *67*, 97 (1960).
[3] H. E. AXELROD, M. G. GULLBERG, and A. F. MORGAN, Am. J. Physiol. *165*, 604 (1951).
[4] B. R. FORKER and A. F. MORGAN, J. biol. Chem. *209*, 303 (1954).
[5] S. MOOKHERJEA and S. C. JAMDAR, Can. J. Biochem. Physiol. *40*, 1065 (1962).
[6] S. C. JAMDAR and S. MOOKHERJEA, Can. J. Biochem. Physiol. *40*, 1059 (1962).
[7] P. B. HAWK, B. L. OSER, and W. H. SUMMERSON, *Practical Physiological Chemistry*, 13th Edn, p. 1071 (1954).
[8] E. F. CALDWELL and E. W. MCHENRY, Archs Biochem. Biophys. *45*, 97 (1953).
[9] H. WADA and E. E. SNELL, J. biol. Chem. *236*, 2089 (1961).
[10] H. B. BURCH, O. H. LOWRY, A. M. PADILLA, and A. M. COMBS, J. biol. Chem. *223*, 29 (1956).