as the fast and the slow component[1,3,5,6] by the slab electrophoresis. Both bands B_1 and B_2 could not be detected in any other organs (liver, kidney, heart muscle and so on) except in nervous tissues. As to species distribution, both bands were observed in almost equal proportion in cat whole brain, while the B_1 component was remarkably dominant in rat as mentioned by previous authors[5]. We carried out micro electrophoresis with micro samples which were prepared from about 1 µg of wet tissues obtained from specific areas of brain (Figure 2). The relative amounts of the two S-100 fractions are determinable directly on the destained micro gels by densitometry in a microdensitometer (MK III CS, Joyce Loebl Co., Ltd.). FILIPOWICZ and associates[3] reported that relative amounts of the two fractions varied from one area to another of beef central nervous system. And a preferential cellular localization of each component in nervous tissues was suggested[11], however, further examinations with microsamples are required.

Recently HYDÉN and LANGE[12] reported an interesting investigation on S-100 protein synthesis occurring in hippocampus pyramidal cells of rat specifically related to learning behavior. In that case micro electrophoretic separation of protein was carried out on polyacrylamide gel (25% w/v) in a capillary tube with Davis's discontinuous buffer system. A front anodal protein band was regarded as the S-100 fraction. However, that band is conceivably one of the multiple fractions of S-100 protein, since electrophoretic heterogeneity of the protein and its dependence upon the buffer system used and the gel concentration has been proved[4,6,7,13].

The present study concerns fractionation of S-100 protein by micro disc electrophoresis as a modified method of slab electrophoresis with continuous buffer system. Disc electrophoresis has the general advantage of electrophoresis on slabs, since electrophoretic patterns are distinct and well reproducible. The micro disc method described here might contribute to neurobiological investigation with regard to learning and other higher brain functions.

Zusammenfassung. Die Trennung von schnellen und langsamen Komponenten von S-100-Protein wurde durch Disk-Elektrophorese mit einem kontinuierlichen Puffersystem ausgeführt und die Anwendungen auf Mikroelektrophorese des sauren Proteins von kleinen isolierten Mengen der Hirnsubstanzen (1 µg Feuchtgewicht) dargestellt.

H. HAZAMA and H. UCHIMURA[14]

Department of Neuropsychiatry, Faculty of Medicine, Kyushu University, 1276 Katakasu, Fukuoka 812 (Japan), and Hizen National Psychiatric Institute, Kanzaki, Saga 842-01 (Japan), 23 August 1971.

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Enzymatic Spectrophotometric Assay for Dihydroorotic Acid in Serum and Urine

Dihydroorotic acid is an intermediate compound produced during de novo pyrimidine nucleotide synthesis. Interest in this and other products of the pathway has increased in recent years as antineoplastic drugs designed to interdict the pathway have been developed and with discovery of an hereditary enzymatic defect of pyrimidine synthesis[1–3]. Quantitation of dihydroorotic acid has been based upon measuring increased absorbance at wavelength 280 nm produced with enzymatic conversion of the compound to orotic acid or upon measuring loss of absorbance at 230 nm with degradation of the compound by alkali[4,5]. These procedures require reading of absorbance in the ultraviolet range making them unsuitable for quantitation of the acid in urine or other biological materials with high background ultraviolet absorbance. The spectrophotometric assay presented is readily adapted for use with urine or serum since absorbance is measured at wavelength 480 nm away from interfering substances absorbing in the ultraviolet spectrum.

Principle of the assay. In the assay dihydroorotic acid of urine or serum is converted enzymatically to orotic acid by dihydroorotic acid dehydrogenase prepared from rat liver. After deproteinization of the reaction mixture, enzymatically formed orotic acid is reacted sequentially with bromine-water, ascorbic acid and *p*-dimethylaminobenzaldehyde to form a colored complex absorbing maximally at wavelength 480 nm[6]. The resulting absorbance is compared to that produced by similarly reacted dihydroorotic acid standards.

Materials and methods. Reagents. 1. L-dihydroorotic acid, 0.01 mg/ml and 1 mg/ml. 2. 0.5 *M Tris* buffer, pH 8.1. 3. 10% trichloroacetic acid. 4. 2.5% *p*-dimethylaminobenzaldehyde in n-propanol. 5. 5% ascorbic acid. 6. Saturated bromine-water. 7. 0.25 *M* sucrose. 8. Enzyme: Dihydroorotic acid dehydrogenase was prepared from rat liver as follows: Rats were decapitated, and the livers were removed immediately and diluted 1:10 wt/vol in 0.25 *M* sucrose at 4 °C. The mixture was homogenized at 4000 rpm for 30 sec in a motor-driven glass teflon homogenizer. The homogenate was centrifuged 15 min at 2000 *g*. The pellet was resuspended in the original volume of the sucrose solution, and centrifugation was repeated. The pellet was then resuspended in half the original volume sucrose solution. This procedure was facilitated by an additional 10 sec homogenization. Dihydroorotic acid dehydrogenase activity of the suspension was assayed using a reaction mixture containing 500 µg L-dihydroorotic acid, 0.5 ml 0.5 *M Tris* pH 8.1, enzyme suspension (0.05 to 0.4 ml) and water to make a final volume of 2 ml. After 10 min incubation at 37 °C, the mixture was deproteinated by addition of 1 ml 10% trichloroacetic acid followed by centrifugation. The blank was identical to the reaction mixture except that trichloroacetic acid was added before

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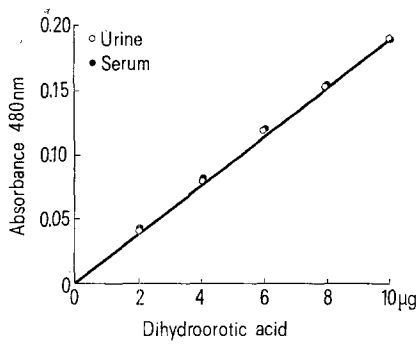
addition of the homogenate. Thereafter 1.5 ml of the supernate was treated as described below for the deproteinated supernate of the urinary dihydroorotic acid assay. Absorbance was determined at wavelength 480 nm, and 1 unit of enzyme activity was defined as that producing a change of absorbance of 0.1 O.D. units under conditions of the assay. The enzyme was found to be stable for at least 3 weeks frozen at $-10°$ C.

Assay of urinary dihydroorotic acid. Urine for the assay was acidified upon collection, then neutralized to approximately pH 7.0 to 8.0 with sodium hydroxide just prior to addition to the assay. Preliminary studies showed that loss of dihydroorotic acid added to urine was negligible when stored acidified at 4 °C for 48 h or at -19 °C for 1 week. Care was taken not to alkalinize the urine excessively since dihydroorotic acid is instable at high pH. The reaction mixture contained 1 ml of neutralized urine, 0.5 ml 0.5 *M Tris* buffer, pH 8.1, 6 units of the rat liver dihydroorotic acid dehydrogenase and water to form a final volume of 3 ml. The mixture was incubated 1 h at 37 °C. The reaction was halted by the addition of 1 ml TCA. Precipitated protein was removed by centrifugation. To 1.5 ml supernate was added 0.2 ml bromine-water. After 1 min 0.2 ml 5% ascorbic acid was added, and after an additional min 2 ml *p*-dimethylaminobenzaldehyde in n-propanol was added. Color was allowed to develop at 37 °C for 15 min, and absorbance was determined at wavelength 480 nm. The blank for the sample was prepared by addition of TCA to the mixture prior to addition of the enzyme reagent. Standards were prepared by substituting for urine known amounts of dihydroorotic acid (5 to 10 µg), and a blank for the standard was prepared by omitting the urine and the dihydroorotic acid. As in the case of the samples, the volumes of the standards and blanks were brought to a total of 3 ml. Calculation of dihydroorotic acid in the urine specimen was based upon the following formula:

$$\frac{(\text{O. D. Sample} - \text{O. D. Sample Blank}) \times \mu\text{g dihydroorotic acid in standard}}{(\text{O. D. Standard} - \text{Standard Blank}) \times \text{ml urine used}} = \mu\text{g dihydroorotic acid per ml urine.}$$

Assay of serum dihydroorotic acid. Blood was collected without anticoagulant and allowed to clot. Serum was separated and stored at 4 °C, or kept frozen at -19 °C when assay was delayed beyond 48 h. The assay using 0.5 ml serum was performed as described for urine.



Recovery of known quantities of dihydroorotic acid added to serum and urine. The values shown represent dihydroorotic acid contained in urine and serum assayed as described. Based upon absorbance produced by the standards, the theoretical recovery values are indicated by the solid line. Also demonstrated is the linear relationship between absorbance produced by the assay and the quantity of dihydroorotic acid in urine and serum.

Recovery of dihydroorotic acid added to the biologic fluids was studied by addition of the acid in the following amounts: Serum, 4, 8, 12, 16, 20 µg/ml; urine, 2, 4, 6, 8, 10 µg/ml. The assay was performed on these samples as described. In addition 6 normal urine samples and 6 normal serum samples were screened for the presence of dihydroorotic acid. Reproducibility of the method was evaluated by adding dihydroorotic acid to serum (10 µg/ml and 20 µg/ml), and 7 separate measurements of the sample were made. Dihydroorotic acid was added to urine (5 µg/ml) and 8 separate measurements of the sample were made.

Results and discussion. Studies of dihydroorotic acid content of urine and serum have been limited by the unavailability of a specific assay method which is free of technical difficulties. Discovery of hereditary orotic aciduria and development of drugs interfering with pyrimidine metabolism suggest applications of a simple, specific assay for the search of other inborn derangements of pyrimidine synthesis and for the study of drugs affecting the pathway. The assay measures as little as 1 µg/ml dihydroorotic acid in urine and 2 µg/ml in serum. Since the acid was undetectible by the assay in these fluids from normal subjects, it is presumably normally present in lower concentrations. Recovery of dihydroorotic acid from normal serum and urine closely paralleled added amounts (Figure). Reproducibility of the method was demonstrated in recovering from serum to which 10 µg/ml dihydroorotic acid had been added an average for 7 determinations of 10.2 $\pm$ 0.2 (SE) µg/ml. With addition of 20 µg/ml of the acid to serum, recovery in 7 determinations averaged 20.2 $\pm$ 0.4 (SE). Recovery of 5 µg/ml dihydroorotic acid added to urine averaged 5.2 $\pm$ 0.1 (SE) for 8 determinations.

For quantitation of the acid, sufficient dihydroorotic acid dehydrogenase activity must be present in the reaction mixture to bring the enzymatic reaction to equilibrium within the hour of incubation. The addition of 6 units of the enzyme should assure adequacy of activity unless an inhibitor is present in the urine or serum tested. The presence of significant inhibition may be excluded by performance of the assay with a mixture of the sample and dihydroorotic acid used in the standard. In the absence of an inhibitor, the absorbance produced by the mixture of the sample and the added dihydroorotic acid should equal the sum of absorbances produced when they are assayed separately. The absence of significant enzymatic inhibition by the urine and serum samples used in this study is shown by the close agreement between added and recovered dihydroorotic acid.

Zusammenfassung. Eine enzymatische, spektrophotometrische Bestimmungsmethode für Dihydroorotsäure im Urin und Serum wird beschrieben.

L. E. Rogers and Kathryn Nicolaisen

Department of Pediatrics,
University of Texas,
Southwestern Medical School at Dallas,
5323 Harry Hines Boulevard,
Dallas (Texas 75235, USA),
7 April 1972.