protein[40-42]. This is credible since it has been demonstrated that highly purified preparations, glycoprotein in nature, with no lipid fraction are capable of initiating the differentiation and subsequent maturation of red cell precursors from primitive mesenchyme.

The above mentioned facts are continually appearing in the literature, and correlate quite closely with the more pertinent features in the present study[12-16,43].

Although the intermediate lobe has not been implicated, heretofore, in the normal sequence of events associated with red blood cell production, this study places the lobe within a central nervous system hormonal complex. It is suggested that under the influence of the hypothalamus, the intermediate lobe undergoes degeneration and autolysis resulting in the formation of intraglandular colloid. The colloid passes into the venous circulation of the cavernous sinuses by way of well defined capsular clefts. Within the circulation, colloid components (possibly ACTH or ACTH-like substances) either directly induce red blood cell production, or activates erythropoietin which initiates this phenomenon. Properly stimulated erythroid committed cells differentiate and proliferate along the red blood cell line.

A working model (Figure) was constructed from these data. The present model is designed to investigate erythropoiesis and makes no attempt to replace any previous models for the regulation of red blood cell production. Although the process or processes concerning the production of erythropoietin are unknown, the model suggests that inactive erythropoietin either stored (kidney, liver, etc.) or circulating, is activated either by a sudden demand for an increased number of circulating red blood cells or by mechanisms regulating the normal turn-over rate of red blood cells.

The model is maintained in a steady state by oxygen sensors. They are sensitive, for example, to a decrease in circulating red blood cells, a decrease in oxygen carrying capacity of blood, a decrease in tissue tension of oxygen, etc. Thus, an immediate demand for circulating red blood cells caused by a rapid shift in the sensors, directly activates erythropoietin or like substances. The operation of the sensors during the normal course of regulating red blood cell production is thought to be through certain hypothalamic centres, which in turn control the cyclic behavior of the intermediate lobe. This results in the formation of intraglandular colloid. Components of the colloid within the circulation activate erythropoietin or like substances. In turn, erythroid committed cells within a large number of organs differentiate and proliferate along the red blood cell line, and at speeds necessary to satisfy the requirements of the sensors. Once accomplished, on the one hand, erythropoietin is inactivated, and on the other, the hypothalamus is inhibited.

Zusammenfassung. Ein Modell zur Untersuchung der Erythropoese zeigt zwei Hauptmerkmale. Erstens aktiviert eine schnelle Veränderung der Sauerstoffsensoren unmittelbar das gespeicherte und das im Umlauf befindliche Erythropoetin. Zweitens wird der normale Verlauf des Umsatzes von roten Blutkörperchen durch die Sauerstoffsensoren beeinflusst, die die Hypothalamuskontrolle des zyklischen Verhaltens des Hypophysenzwischenlappens und seiner innerdrüslichen Kolloidproduktion steuern.

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PRO EXPERIMENTIS

Separation of Fast and Slow Components of S-100 Protein by Column Electrophoresis with Continuous Buffer System and Application to Micro Electrophoresis

The S-100 protein is a defined and brain specific protein. Correlations between the protein and neural functions are supposed but not yet certain. For the physiochemical study of neural functions, micro analytical methods at the cellular level are valuable because of the highly differentiated structure and function in the central nervous tissue.

With regard to the separation of the S-100 protein, UYEMURA et al.[1] tried slab electrophoresis of mixed agarose acrylamide gel with continuous buffer system and obtained much better results in comparison with that of starch gel electrophoresis[2]. According to the former electrophoretic systems, brain S-100 was separated into a fast and a slow migrating fraction. Identifications of the fractions have been detailed[1,3-7]. In the present paper, an application of the method to micro disc electrophoresis is described.

Materials and methods. Cats were anesthetized with sodium pentobarbital and rats were killed by decapitation. The brains were rapidly obtained after extensive craniotomy and separated respectively into forebrain, brain stem and cerebellum. These brain samples were homogenized in a Teflon homogenizer with 2 volumes of *Tris*-phosphate buffer 5 m*M* (pH 7.1) and centrifuged at $10,000 \times g$ for 30 min. Before use the supernatant was colored with a small amount of bromphenol blue (BPB) and used as a sample for electophoresis. Extracts from other organs of cat were prepared in the same way. In order to obtain micro samples for micro electrophoresis fresh cells or small pieces (approximately 1 µg of wet wt.) of tissue in identified locations were dissected from brain slices (1.5 mm

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thick) with pointed stainless steel wires under a stereomicroscope as described elsewhere[8]. These micro samples were homogenized for 2 min in a 440 μm capillary tube (2.0 μl in content) containing 1.0 μl of *Tris*-phosphate buffer 5 m*M* (pH 7.1). A loop of thin (30 μm in diameter) stainless steel wire fastened to a high-speed dental drill (20,000 rev./min) was used for homogenization. The homogenate was centrifuged at 15,000 × *g* for 15 min and the supernatant (0.5 μl) was used for micro electrophoresis.

Mixed agarose (0.75% of the final concentration) acrylamide (various final concentrations from 3 through 20%) gels buffered to pH 8.6 with 0.09 *M* *Tris*-acetate buffer containing 2.5 m*M* EDTA were prepared at 60 °C by a modification[9] of the procedure of URIEL[10]. A 5 mm diameter plastic tube was used for standard disc electrophoresis and 2 kinds of glass capillary were used for micro disc electrophoresis; one was 1.0 mm in diameter and 49 mm in length with a 40 μl capacity and the other was 440 μm in diameter and 33 mm in length with a 5 μl capacity (Drummond Sci. Co., Broomall, Pa.). After the gel was completed in the tube (or the capillary) 40% sucrose was introduced approximately 2 mm (or 1 mm for the capillary) in thickness upon the gel. Then a tissue sample was placed 2 to 3 mm (or 1 to 1.5 mm for the capillary) in thickness to make an even layer on the sucrose solution. Thereafter the electrode buffer (0.09 *M* *Tris*-EDTA acetate buffer, pH 8.6) was filled up carefully by means of a thin micropipette without disturbing the even sample layer purpled with BPB. The standard electrophoresis in a 5 mm diameter tube was run with a current of 3 mA for 90 min until BPB moved 7 cm. For micro electrophoresis it is important for good separation of S-100 fractions to use a relatively intensive current per gel column, that was 700 μA for 20 min to a 1.0 mm diameter capillary and 90 μA for 15 min to a 440 μm diameter capillary. After electrophoresis the gel extruded with a tightly fitting wire was stained overnight with 0.013% Coomassie brilliant blue R 250 (Kishida Chemicals Co., Ltd., Osaka) dissolved in methanol = glycerol = acetic acid = water (40 = 2.5 = 5.5 = 52, by vol.) and destained using methanol = glycerol = acetic acid = water (20 = 2.5 = 5.5 = 72, by vol.).

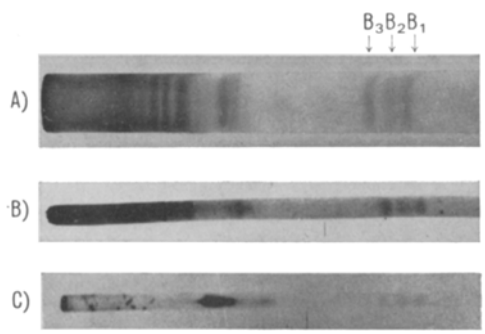

Fig. 1. Disc electrophoresis with continuous buffer system on mixed agarose (0.75%) acrylamide (15%) gel of soluble protein of cat brain stem. B_1 and B_2 bands are the fast and the slow components of S-100 protein, respectively. A) standard electrophoresis in a 5 mm diameter plastic tube. B) semimicro electrophoresis in a 1 mm diameter glass capillary (× 2). C) micro electrophoresis in a 440 μm diameter glass capillary (× 5).

Results and discussion. Three fast components were found at the region of the migrating front designated as B_1, B_2 and B_3 from the anodal end. The 3 bands were perfectly separated from each other on a 15% acrylamide and 0.75% agarose composite gel (Figure 1). On the occasion of micro electrophoresis rear bands were not always well fractionated. This result came from the intensive current used for gaining good separation of the three fractions from each other. The bands of B_1 and B_2 were placed just in front of the migrating line of BPB on a 5% gel and not completely differentiated on a gel under 5% of concentration, while the B_3 could be separated well from the others even on a 3% gel. These findings were coincident with the previous result[1] from the slab electrophoresis using mixed agarose acrylamide gel, and accordingly the B_1 and B_2 here were considered to be fractions of S-100 protein. Moreover the S-100 bands separated on a gel slab according to UYEMURA et al.[1] were re-run on our column. Consequently the B_1 and B_2 fractions were identified, respectively,

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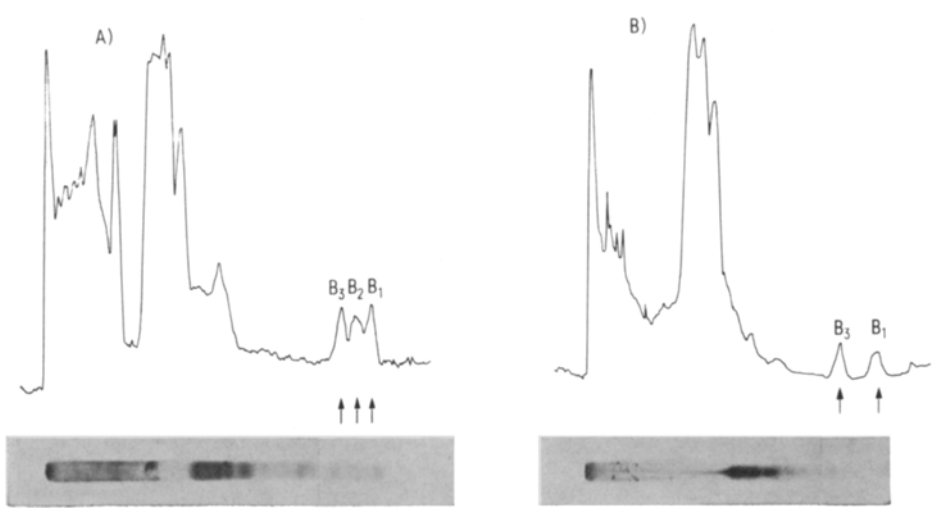

Fig. 2. Micro electrophoretic pattern and corresponding microdensitometric recording. Microsample was obtained from pyramidal cell bank (CA 3 region) of A) cat and B) rat hippocampus (see text). Micro electrophoresis was carried out in a 440 μm diameter capillary and the destained gel was scanned directly with a microdensitometer. Final magnification of the photographs and the original curves is × 10. B_1 and B_2 bands are the fast and the slow components of S-100.

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.

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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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