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A NEW ULTRAMICRO ANALYSIS OF PROTEIN BY S.D.S.¹ POLYACRYLAMIDE GEL ELECTROPHORESIS

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The physiological mechanism of protein excretion in the urine, especially that in the nephron remains unexplained. If the method of analyzing proteins in a small specimen collected from the nephron by a micropuncture technique could be further developed, the mechanism of the protein excretion into the tubular fluid would be greatly clarified.

The volume of the tubular fluid collected by the micropuncture technique is merely a few nanoliters. Such being the case, experiments were undertaken to devise a highly sensitive method for determination of a few nanograms of proteins in even a small specimen. The principle of our method is a microdisc electrophoresis of fluorescent proteins. The procedures were divided into two main parts. The first step was preparation of a fluorescent protein contained in a specimen applying a reaction with a reagent, 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl), (1). The second step was microdisc electrophoresis of the fluorescent proteins.

DNS-Cl reacts with the N-terminal of protein structure and gives off a strong fluorescence for protein under a weak alkali solution.

For microdisc electrophoresis, glass capillaries, 32 mm long and 290 μ in diameter, were used. The volume of the capillary was 2 μ l and such capillaries are commercially available (Microcaps Drummond Scientific Co., U.S.A.). The inside of the capillaries should be carefully washed and siliconized using 2% dimethyldichlorosilane.

Gel and buffer solutions were prepared according to the method of Weber et al (2). Ten percent polyacrylamide gels containing 0.2% sodium dodecyl sulfate (S.D.S.) were used for the molecular weight determination of proteins in a specimen.

The practical procedures are as follows:

(a) The specimen containing proteins is mixed with the same volume of 1 M triethylamine bicarbonate buffer (pH 7.4) and of 4% Dansylchloride Cycloheptaamylose solution (D.C.I.C.) containing 0.1 M phosphate buffer solution (PBS) and of 6 M urea. D.

1 S.D.S.: sodium dodesyl sulfate.

C.I.C. is an abbreviation of Dansylchloride Cycloheptaamylose Inclusion Complex (Kinoshita, T, personal communication). DNS-Cl has very low solubility in water, but, D.C.I.C. has higher water solubility than DNS-Cl and the increase of water solubility produced good results for the electrophoresis, although the mechanism could not be elucidated.

Practically, the volume of specimen to be used depends on the protein concentration. If the specimen contains more than 10 mg% concentration of proteins, a volume of 5~6 nanoliters is adequate for the determination.

The procedure must be done throughout under a microscope using a manipulator.

It is very important, however, that this procedure of mixing be carried out on the gel of polyacrylamide in the capillary (See Fig. 1). This capillary was next incubated at 37°C for two hr in a moist chamber, in order to label the protein by DNS-Cl.

FIG. 1. Schematic drawing of arrangement for ultramicro disc electrophoresis

- a. upper reservoir
- b. gel tube
- c. lower reservoir

After dansylation, the capillary was set again under microscope and the same volume as specimen of 0.01 M PBS containing 2% SDS was further added to the mixture. The capillary was re-incubated at 37°C for 30 min. After the second incubation, gel buffer (0.01 M PBS, 0.2% in SDS) diluted 1:1 with water was carefully made to cover the top of the sample mixture and the capillary was filled up with this buffer solution.

(b) Electrophoresis was performed in this capillary applying the constant electric current of 15 μ A. Under these experimental conditions, byproducts such as DNS-OH, DNS-NH₂, and label amines, aminoacid, peptides of small molecular weight were moved also, but faster than labelled proteins. During electrophoresis, DNS-derivatives, such as

FIG. 2. Comparison of ultramicro disc electrophoresis
 a. ultramicro disc electrophoresis of Newt serum capillary I.D. 0.29 mm, containing protein : 9×10^{-8} g
 b. macro disc electrophoresis of Newt serum tube I.D. 6 mm, containing protein 1×10^{-3} g

the byproducts and DNS-proteins were visible as yellow bands under an ultraviolet lamp. Therefore the time for electrophoresis was controlled by the lamp and the electrophoresis was stopped when the front band reached within 2 cm from the top of the gel. Usually the time for electrophoresis was about 1.5~2.0 hr. The capillary was then placed on the stage of the scanning fluorometer which had been specially constructed for this purpose and the intensity of the fluorescent bands in the gel was recorded.

In order to check the accuracy of this method, the results were compared with those using ordinary macromethod. As a specimen, newt serum was used (3).

Fig. 2, shows both patterns of electrophoresis. In the macromethod, the serum of 10 μ l (1000 nl) was used. On the other hand, only 3 nl was used in the microdisc electrophoresis. The pattern of the fractionated proteins using the ultramicro method differed slightly in detail from that of the macromethod, especially the dimensions of each small curve which indicate the concentration of each protein contained in the specimen. However, the proteins were fractionated in the same mode, such as shown in figures, indicating the existence of various proteins. These facts suggest that although it is possible to view many different proteins in a specimen when the ultramicro method is used, the method for determination of the concentration of each fractionated protein requires further development.

According to Crossbach (4), Hyden et al (5), Neuhoﬀ (6) and Oken (7, 8), disc electrophoresis is most adequate for the measurement of very small samples of proteins. In these experiments, however, the gel which contains the fractionated bands of proteins was separated from the glass capillary and stained with dye at the outside of the capillary. The capillary tube is so small that separation of the gel is difficult. Further, the location of the fractionated protein bands could be disturbed during the separation and the gel dehydrated during the course of recording.

In our method, the gel was kept in the glass capillary throughout the experiment, therefore the dye staining procedure could be omitted.

As Weber and Osborn (2) reported, SDS polyacrylamide gel electrophoresis was useful for the determination of molecular weights of proteins by calculating rates of mobility. Inoue (9) has also reported the method of electrophoresis concerning DNS-proteins. This author used acetone to dissolve the DNS-Cl. Acetone was, however, not suitable for the micromethod, as the proteins tended to aggregate or polymerize. For this reason, we used D.C.I.C. which is soluble in water and did not affect any change on proteins.

The scanning fluorometer constructed especially for this purpose was set to scan a 200 μ diameter slit and the speed was 20 mm/min. If the slit were set narrower and the speed adjusted to a slower rate, the sensitivity of the equipment would be greater and amounts of proteins less than 10^{-9} g could be detected.

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NALOXONE-INDUCED ABSTINENCE SYNDROMES IN MORPHINE-TREATED MICE¹

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Hano et al. (1) suggested that it was difficult or almost impossible to demonstrate physical dependence development in mice. Maggiolo and Huidobro (2), on the contrary, reported that the abstinence syndrome in mice was rich in manifestations such as a very intense increase of the spontaneous activity and jumping behavior, but unfortunately a quantitative evaluation of its intensity in order to establish comparisons in morphine pellet implanted mice was extremely difficult.

Recently, Way et al. (3) have succeeded in quantifying the intensity of physical dependence by estimating the dose of naloxone necessary to precipitate withdrawal jumping response. Saelens et al. (4) also proposed that this procedure could be used as a screen-

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