APPLICATION OF DISC ELECTROPHORESIS TO THE STUDY OF BRAIN PROTEINS

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Disc electrophoresis, first introduced by ORNSTEIN[1] and DAVIS[2], has become a useful tool in biochemistry, especially when only a small amount of protein in dilute solutions is available for study. The technique has also been successfully combined with radiometry and has been modified to a microscale for the separation of proteins in quantities of the order of 10^{-8} g[3].

Disc electrophoresis with increasing concentration of the gel[4] was used to study the acidic proteins, specific for the brain, which were originally described by MOORE AND MCGREGOR[5]. By this procedure the protein front of the separation, containing the immunochemically definable acidic protein(s), could be separated into three and finally, five components. Three of these, called bands "a", gave precipitin bands with the antiserum. The "b" fraction had the same rapid metabolic turnover as the "a" component but gave no precipitation with antiserum. The "c" band had a slower turnover and comprised almost 80 % of the proteins of the front band in a 7.5 % gel. This band also produced no immunochemical reaction with the antiserum against the acidic brain protein.

The present work deals with the application of disc electrophoresis to the study of turnover rates of acidic brain proteins in normal rats. In a following paper results will be presented from a study of brain proteins of rats treated with tranylcypromine.

MATERIAL AND METHODS

Female albino rats of Sprague-Dawley strain weighing between 200 and 230 g were used in these experiments. L-Leucine-4,5-T (23 C/mmole, The Radiochemical Centre, Amersham, Bucks., England, and 5 C/mmole, New England Nuclear Corp., Boston, Mass.) was injected intraperitoneally in doses of 200 μC/100 g body weight.

The techniques used for perfusion of the animals, extraction of soluble brain stem proteins, electrophoresis in acrylamide gel, and radioactivity measurements, were as previously described[4] and only a few additional details will be given:

The perfusion of the animal and the preparation of homogenates were carried out in a cold room at +4°. The brain stem was isolated by an upper section at mesencephalic level between the cerebellum and the cortex, just below the posterior colli-

culus, and a lower section at the boundary between the brain stem and the spinal cord (Fig. 1). Cerebellum was removed by section of the cerebellar peduncula. Brain stem samples—weighing about 200 mg—were quickly homogenized in 4.5 ml of 0.25 *M* cold sucrose + 0.5 % Triton-X-100. The homogenate was stored for 6 h at —20°, then thawed and centrifuged at 100,000 *g* for 60 min. After determination of the protein content[6], 0.15 ml of Tris-buffer[2] was added to the supernatant for each milliliter of extract, and was kept at —20° until used.

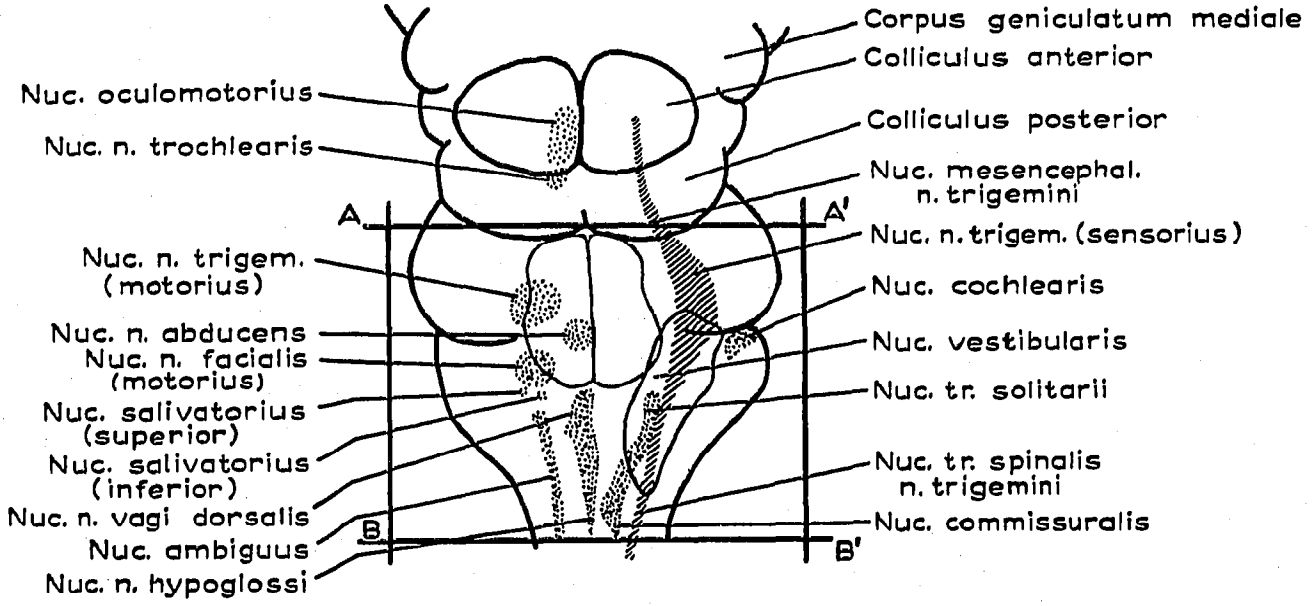

Fig. 1. The brain stem of rat with limitation of the sample area denoted. (From: W. ZEMAN AND J. R. M. INNES, *Craigie's neuroanatomy of the rat*, Academic Press, New York, 1963).

Disc electrophoresis was usually performed in 11.2 % acrylamide gel, in glass tubes with an inner diameter of 7 mm. The sample was run directly in 11.2 % gel instead of using the "double-running" technique[4] (see below, discussion). The pH's of the stock solutions were: 6.9 for the sample and spacer buffer, 8.9 for the lower gel, and 8.3 for the electrode buffer. The gels were stained for 90 min in 0.5 % amidoblack in 7.5 % acetic acid. Excess dye was removed by repeated washing with 7.5 % acetic acid. Acetic acid washing was preferred to electrophoretic destaining as suggested by some authors[2,7,8] and produced more consistent results.

Four parallel gels were run for each sample. The individual protein bands were cut out and placed separately onto pieces of filter paper (the four slices of the same band were pooled), combusted and the radioactivity determined[4]. For each count of the samples a parallel count of the standard was performed in order to determine the efficiency of the apparatus for every sample. At the onset, a standard calibration curve was made for the external counting correction. Prefixed counts were set so as to have a standard error ($\varepsilon d\,\%$) of 3 % for each determination. Samples were counted for 10 min, the "k" value (ratio between the activities of sample and background) calculated and the counts ("x") preset using the following formula[9]:

$$x = \frac{10^4(1 + 1/k^2)}{(\varepsilon d\,\%)^2(1 - 1/k)^2}$$

The statistical analysis of the results was performed by means of variance and co-variance analyses (Snédécor's F test).

A densitometric reading of the gels was also performed in order to have a semi-quantitative evaluation of the electrophoretic pattern and to check the reproducibility of the separation technique used. The photographs were taken using a reflex camera, Kodak panatomic film 24 × 36 mm (40 ASA), a lens-to-gel distance of 40 cm, diaphragm $f = 22$, exposure time 1/2 sec. A 60 W tungsten lamp was used with an opalescent glass interposed between lamp and gel. The bulb of the lamp is large enough to give an uniform illumination in the area of the three fast moving bands (while for the whole gel a fluorescent tube could be used).

Recording of the negative film was done by a Joyce-Loebl microdensitometer, with a 50 mm objective and a gray wedge with a density interval of 1 D (optical density unit). The film used has a characteristic curve (density–log exposure) which is also linear between the range of 1 optical density unit, and this was the maximum interval found in the three front bands of a 11.2 % gel stained with amidoblack.

Staining and destaining times were chosen so that external conditions during these procedures were kept strictly constant. Recording of the gels in the Joyce-Loebl microdensitometer was performed with a slit height of 2 mm (corresponding to the width of the image of the gel on the negative film). The width of the slit was kept as nearly constant as possible and large enough to avoid interfering effects from individual silver grains of the emulsion.

TESTING AND EVALUATION OF THE PROCEDURE

Tissue homogenization

It is well known that the way in which the extraction is carried out is critical for the recovery of proteins[10]. Therefore, as a first step, the same glass homogenizer was used in all the extractions, in order to avoid any variation in the clearance between the pestle and the wall of the homogenizer. It was empirically found that a clearance of 70 μ gave the best results in protein extraction.

As regards the extraction medium, 0.25 M sucrose + 0.5 % Triton-X-100 was found to give the best results in brain stem homogenates (Table 1). Extraction without

TABLE I

EFFECT OF THE EXTRACTION MEDIUM ON THE TOTAL AMOUNT OF SOLUBLE PROTEIN IN THE SUPERNATANT AFTER CENTRIFUGATION AT 100,000 g FOR 60 MIN

	(*a*) *Brain stem* *22.5 ml/g of fresh tissue*			(*b*) *Brain* *2.5 ml/g of fresh tissue*	
Extraction medium	0.25 M sucrose + 0.5 % Triton-X-100	0.25 M sucrose	Water	Bicarbonate buffer + 0.5 % Triton-X-100	Bicarbonate buffer
Protein (γ/200 μl)	450 ± 9.6	226	151 ± 19	397	342
Number of samples	8	1	3	2	2

Triton gave 50 % less yield of protein. When only water was used, the yield was one-third of that obtained with sucrose and Triton. For brain protein extraction, a bicarbonate buffer, pH 7.3, was also employed as described by BERGMANN AND SEGAL[11]. Addition of Triton-X-100 to the bicarbonate buffer improved the protein extraction. However, the effect of Triton was less marked than in the case of sucrose media because of the high concentration of extract required for enzyme studies (2.5 ml of solution per gram of fresh tissue, while the optimum should be a volume of extraction medium at least five times the volume of the fresh tissue[10]).

Filling of sample

In these experiments, Sephadex G-25 and G-100 have been used instead of the 4 % upper gel suggested by DAVIS[2] in the original technique. A mixture of Sephadex G-200 and sucrose instead of the upper gel was first used by BROOME[12] for equine haemolysate and by SULITZEANU AND GOLDMAN[13] for immunochemical studies.

It should be noted, however, that the use of Sephadex modifies the sieving properties of the system. The frictional resistance increases as the molecular size of the protein particles decreases in the range of the Sephadex filtration capacity. As a final result, the order of the "steady-state stacking"[1] will be reversed and the small proteins will have to pass through a filter of the big ones before entering the lower gel. This could be an additional advantage in the use of Sephadex, provided the concentration of the sample is kept constant (for further details see section "Recovery and protein pattern").

Polymerization of the upper gel could take place when it becomes necessary to use a large volume of diluted sample. The concentration of stock solutions, used for the gel, should be increased by subtracting from them the volume of water contained in the diluted sample, in order to keep constant the final concentration of the mixture[2]. But the frictional problem would not be solved because the theoretical viscosity, which is a function of the particle radius[1], increases further with increasing protein concentration in the spacer gel, when the proteins are packed in a thin disc.

It is advisable not to fill the tube completely with Sephadex plus sample, in order to minimize the loss of proteins by convection. A column of about 3 mm should be kept free on top of the tube and filled with a layer of Sephadex. When this layer of Sephadex was replaced by electrode buffer, the resistance of the column was higher. The intensity of the current decreased during the run, while it was steady when only Sephadex was used and the electrode buffer was only in the upper main reservoir (Fig. 2). A constant-voltage power generator was used and the intensity of the current continuously adjusted to 2 mA for each 7 mm gel.

The general law of electrical resistance for a non-ohmic conductor is $i = (V + E)/r$, where E is the input or output of energy into the system. When the intensity is kept constant, a change in the voltage may depend on a change both in the resistance (r), or in E. In the case of disc electrophoresis, E is the energy required to move the ions through the column. In the present experiments, the value of E apparently did not change since the migration velocity of the front band was constant and independent of the height of the buffer column. Thus, the resistance decreased during the run mostly because of the mixing of two different buffers with different pH's (6.9 and 8.3). Such buffer mixing results in a loss of sharpness of the boundary between the trailing and the leading ions.

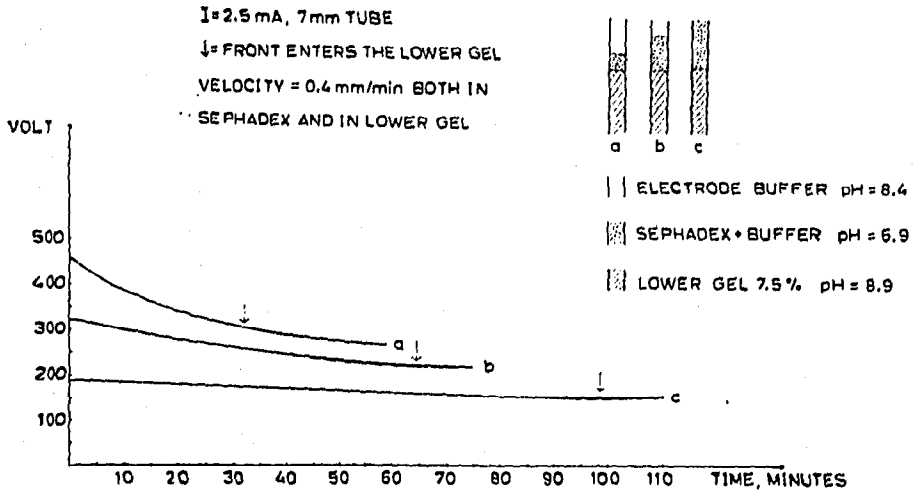

Fig. 2. Effect of electrolyte amount in tubes on voltage.

The degree of polymerization of the gel

This is one of the most critical steps in acrylamide-gel electrophoresis[2]. It is influenced by many factors. It is essential that the gel is always polymerized, as far as possible, under the same conditions (time, light source, room temperature, humidity, temperature of stock solutions when they are taken out of the refrigerator for use, etc.). Different electrophoretic patterns were obtained from the same sample with gels at various stages of polymerization obtained by changing the proportion between Temed and ammonium persulphate in the stock solutions. The less the polymerization is advanced, the more pronounced is the swelling of the gel when it is placed in 7.5 % acetic acid for destaining. The measure of the distance between the front band and the starting point of the gel, before and after swelling, gives a reliable indication of the degree of polymerization.

The maximal amount of protein

The maximum amount of protein that could be used to achieve the best separation is 200 γ in tubes of 5 mm inner diameter as suggested by DAVIS[2], (or 400 γ in the 7 mm tubes used in this experiment). For separation in a 7.5 % gel, 400 γ is the proper amount in order to obtain sufficient protein yield in each band to permit radioactivity determination. In cases where the 11.2 % gel was used, 400 γ of protein was insufficient because too little protein entered the gel and a satisfactory separation of protein fractions was not obtained. This inconvenience can be avoided in two ways. The first is to run a large volume of sample in 10 mm inner diameter tubes (7.5 % gel; sample 1000 μl = 2000 γ of protein), cut the front band without fixing and staining, and re-run this band in 11.2 % gel, according to MCEWEN AND HYDÉN[4] following the advice of VOS AND VAN DER HELM[14]. The second possibility is to run the sample directly in 11.2 % gel. If the amount of protein is too large—more than twice the maximum amount suggested by DAVIS[2]—the separation is not satisfactory (Fig. 3). Each band may split because when one part of the protein enters the lower gel, the other part is still concentrating in the upper Sephadex layer. With the labelling technique used in these experiments, about 600 γ of soluble protein in a 7 mm tube and 11.2 % gel was found to be the appropriate amount in order to avoid the doubling of the bands and to afford enough protein in each band for measurement of radio-

activity in the four gels with a standard error of 3 %. A comparison of the electrophoretic patterns thus demonstrated that both these procedures gave a satisfactory result. An accurate study of the quantitative relationship between the two techniques is in progress.

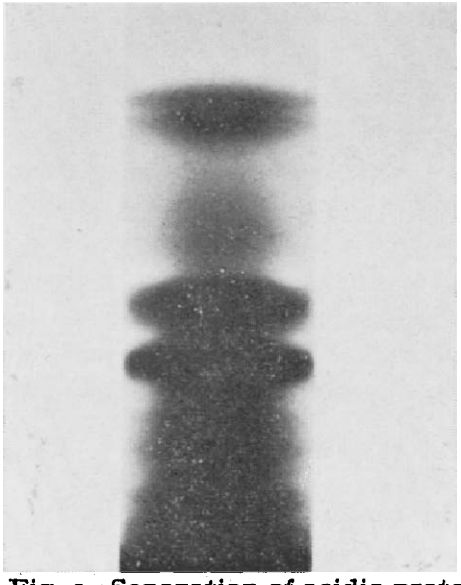

Fig. 3. Separation of acidic protein discs 0, 1 and 2 into double discs in 7.5 % polyacrylamide gel. 2000 γ proteins applied, which is five times the normal optimal amount.

Albumin interference

In these experiments the animals were perfused in order to remove as much blood serum in the sample as possible, although perfusion will not remove blood completely. The albumin does not interfere with the three fast moving bands, because it has been found that its mobility is lower than that of band 2 in 7.5% gel. Prealbumins may interfere as they are present adjacent to the rear of the front band[2]. For this reason it is important to have constant perfusion conditions, although the amount of prealbumins eventually present is too low to give any significant error.

Recording

For densitometry, BURNS AND POLLACK[15] used bent tubes and a Polaroid camera. With this technique, the lens-to-gel distance should not exceed 155 mm for the reading of the photographic plates on a macrodensitometer. In the present study, a 24 × 36 mm film, Kodak panatomic 40 ASA, was used and the geometrical unsharpness was avoided by using the Joyce-Loebl microdensitometer. A study of the trigonometric relationships showed that the two discs near the ends of the gel, which were separated by less than 0.3 mm with a migration front of 32 mm, will not be resolved. A distance of 0.3 mm between two bands is an exceptional occurrence, and below the limits of the optical resolution.

The grain effect could be satisfactorily avoided provided the opening of the slit in the scanning apparatus was not too small.

The maximum value of absorption in a disc acrylamide-pherogram of serum is between 4 and 5 optical density units[16]. The maximum value of 4–5 D is found only in the peak of albumin in serum, while a range of less than 2 D covers the absorption range of all the proteins except the albumin peak. In the present study, only the

three fast moving bands have been taken into account, and an interval of 1 optical density unit was found to be the optimum for this procedure.

Recovery and protein pattern

When the amount of the protein in the sample is changed, it should be stated whether the quantity of protein in each band is still proportional to the degree of dilution. An experiment was carried out to test the relationship between the total amount of soluble protein in the sample and the recovery of proteins in the first three bands. The determinations were performed by densitometry and radioactivity counting. It was found that when the protein concentration was decreased (in the same volume of sample) the protein content in some discs was not proportional to what could be expected. This should be noted, especially when proteins from various sources and with different concentrations have to be compared. A detailed study on this subject will be published elsewhere[17].

Between the 0 and 1 bands in a 7.5 % gel, a very small protein fraction was faintly visible when a large amount of sample (530 γ) was used. It is not surprising that some protein bands containing a very small amount of protein should give an unsatisfactory staining reaction. Such bands, however, can be discerned due to their ability to scatter light falling obliquely incident on the gel. It has also been shown that when a gel is stained for enzyme reaction[18] the bands giving a positive enzyme reaction do not strictly correspond to the bands stained for protein with amidoblack. The presence of the small protein fraction between bands 0 and 1 becomes important when the first band 0 is cut out for re-running or for radioactivity counting, since some undetectable protein may remain in the upper or in the lower part of the section and alter the results.

Reproducibility

Reproducibility of the technique was satisfactory as shown by the results in Fig. 4. Densitometric curves from two gels with the same protein sample

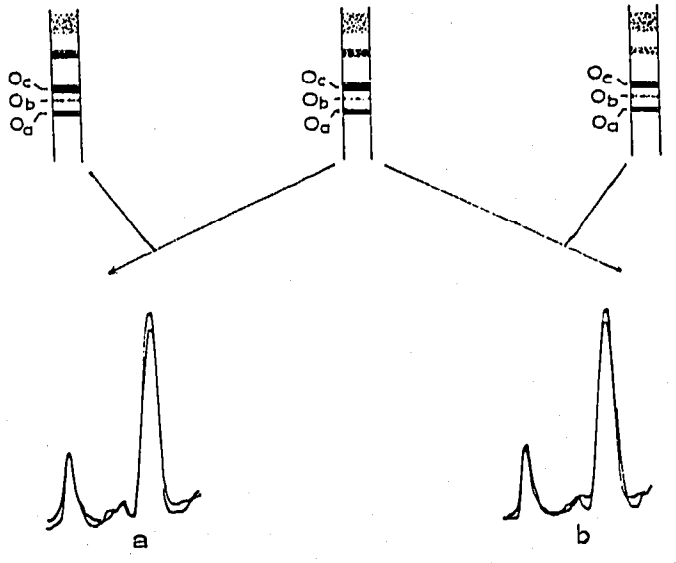

Fig. 4. Protein separation in 11.2 % gel to test the reproducibility of the method: (a) two densitometric recordings from two gels with the same protein sample; (b) two densitometric recordings from protein samples from two animals.

(Fig. 4a) and two samples from different animals (Fig. 4b) gave consistent results.

The relative mobility of the three fast moving bands in a 11.2 % gel and 600 γ of protein per 39 mm^2 section of the tube (7 mm diameter) is as shown in Table II.

TABLE II

RELATIVE MOBILITY OF THE THREE FAST MOVING BANDS

Band	*Relative mobility units**
oa	1.23
ob	1.11
oc	1.00

* The "*r*" value has been related to band oc.

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SUMMARY

Various steps in disc electrophoresis of brain stem proteins have been studied with special regard to separation of proteins on 11.2 % gel and in 7 mm tubes.

The best extraction medium tested was found to be 0.25 *M* sucrose with 0.5 % Triton-X-100. The use of Sephadex as an anticonvection medium and the related electrical properties of the system have been taken into consideration. Controlled gel polymerization gave a consistent protein pattern. Optimal amounts of protein/sample for separation on 11.2 % gel are given. Densitometric curves were recorded from negative films with a Joyce-Loebl microdensitometer. The reproducibility of the technique and the experimental results are discussed.

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