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# MICRO-ELECTROPHORETIC DETERMINATION OF PROTEIN AND PROTEIN SYNTHESIS IN THE $10^{-9}$ TO $10^{-7}$ GRAM RANGE

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SUMMARY

A procedure is described of electrophoretic separation of $10^{-7}$ to $10^{-9}$ g of proteins on polyacrylamide gel in 200 $\mu$ or 400 $\mu$ diameter glass capillaries. Specific activities of tritium-labeled protein fractions are determined by a combustion technique in combination with interferometry.

To be able to compare protein specific activities in identical structures of the same organism or of different animals, a procedure is described to correct for variations in the concentration of the precursor amino acid.

An application of this method to a neurobiological problem is described.

Most organs in the body are composed of several types of cells some of which may dominate in the expression of the function of the organ. The heterogeneity of the tissue composition requires the use of extraordinary methods of sample extraction and analysis.

The requirements are usually met by any method which allows the analysis of substances in amounts of $10^{-7}$–$10^{-9}$ g in a volume of $10^5$ $\mu^3$ provided pure samples of the various cell types can be obtained.

The present paper describes how micro-disc electrophoresis of labeled proteins in brain cells, nerve cells or glia can be used to assess the type and turnover of soluble proteins in comparable loci of the same brain or in identical structures of different brains. The specific activities obtained are corrected for variations in the local concentration of the precursors. The new procedure is a further development of a method for micro-disc electrophoresis which we have previously published[1].

## SAMPLING OF CELL MATERIAL

### *Isolation of nerve cells and glia*

The animals are killed rapidly and bled and the brain is immediately removed. A section is made through the part of the brain which is of interest. This section, 2 to 4 mm in thickness, is placed in a solution of 0.25 *M* sucrose or Krebs-Ringer solution, and on the cooling-stage of a stereo-microscope. This is provided with 20 × oculars

and an auxiliary 2 × front lens, giving a magnification of 20 × – 160 ×. The light sources are provided with infrared filters to remove heat.

The brain section is carefully kept under solution and flooded with a 0.01 *M* solution of methylene blue for 20 sec. It is then washed and kept under pure sucrose or salt solution. This treatment will render the nerve cell bodies slightly blue for at least 5 min. After that time, the staining may have to be renewed. Even if this is not done, the cells are still recognizable to the trained eye against the glistening white glia because of their yellowish appearance and because the synaptic knobs surrounding the cell border keep the stain. This has been described previously[2].

The cells, together with part of the surrounding glia, are lifted from the section by inserting a pointed stainless-steel wire (Nicrothal L, AB Kanthal, Hallstahammar, Sweden) 18 to 28 $\mu$ diameter, below the cell. Each cell is lifted out of the section and placed in Krebs-Ringer solution. This is done by free hand manipulation. The stereomicroscope should be provided with hand supports.

The nerve cell bodies plus a considerable part of the dendrites, free swimming, are freed from the adhering glia by gentle manipulation from below. Fig. 1 shows six isolated nerve cells photographed in incident light. Note the absence of glia and the length of the dendrites. When these become too thin they break off, but evidently repair the holes immediately. (See below). A careful study of the time required to isolate ten nerve cells already placed in an isotonic solution from the surrounding glia showed it to take around 2 min.

When the glia is removed from the cell body plus processes, they have a tendency to stick together, especially in an isotonic ion solution. It is then easy to shape the glia into a clump containing 7–8 nuclei of about the same volume as that of the nerve cell

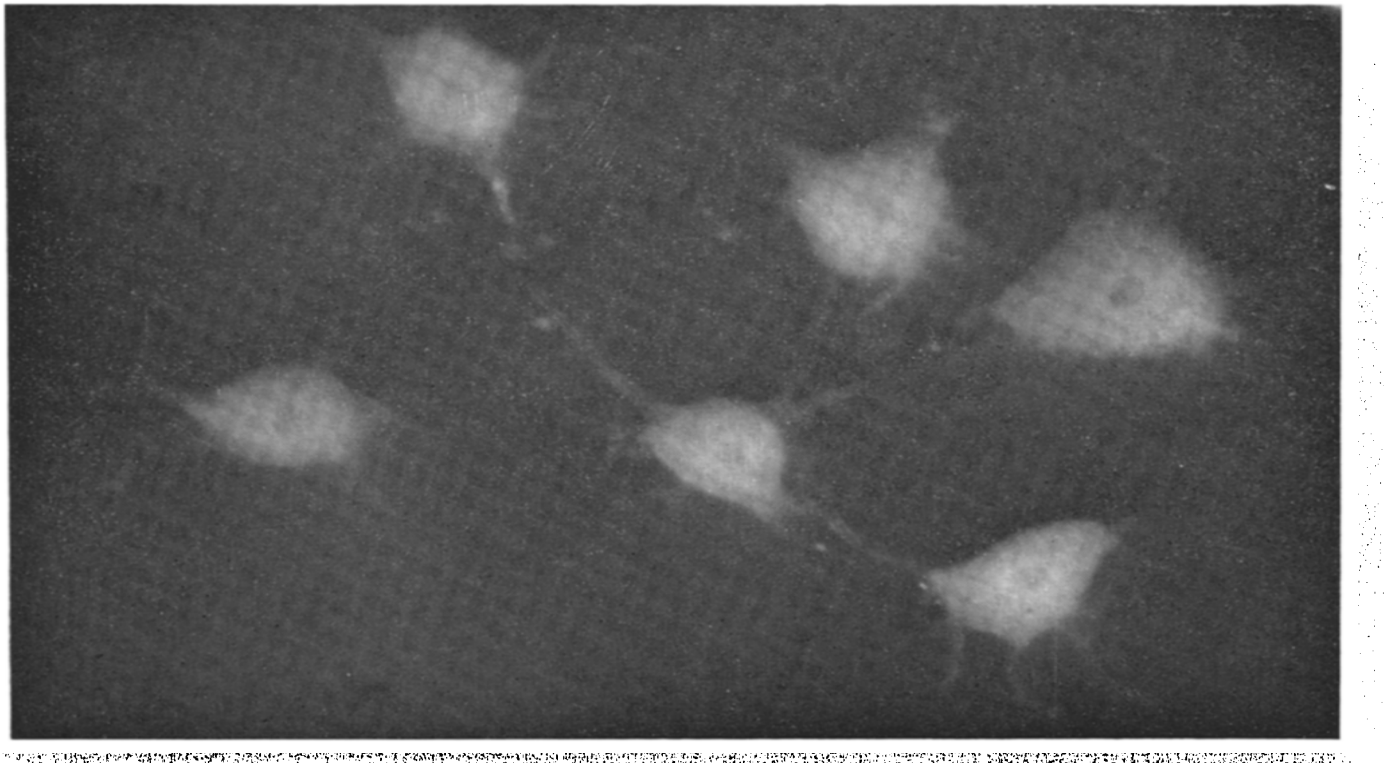

Fig. 1. Six nerve cell bodies including the first part of the dendrites, unstained and freed from surrounding glia by free hand dissection and photographed in incident light. Magnification 100 ×.

for the gel. The refractive index of the embedding medium is $n_m$. Thus, the general expression for the displacement (optical path difference) within a protein disc is

$$F = d_g(n_g - n_m) + d_p(n_p - n_m)$$

from which we want to calculate $d_p$ in order to determine the amount of protein. This is done most simply by first measuring $F_g$ in the protein free gel near the protein disc and then $F$ within the disc. Thus $F_p = F - F_g = d_p(n_p - n_m)$. If the value of $n_p$ (often near 1.60) is known, $d_p$ can be calculated. If not, the gel is embedded in two media with different refractive indices and we thus obtain:

$$F_{p1} = d_p(n_p - n_{m1})$$

$$F_{p2} = d_p(n_p - n_{m2})$$

from which both $d_p$ and $n_p$ can be calculated.

The optical path difference is measured practically by means of the so-called trace displacement method which is schematically illustrated in Fig. 7. It should then first be observed that we do not have to measure the displacement of the protein disc relative to the embedding medium but relative to that of the gel. Fig. 7 schematically shows part of a micro-gel with a protein disc in the interference microscope. Since

Fig. 6. Interference micrographs of a 200 $\mu$ diameter gel embedded in benzyl alcohol–glycerol, $n = 1.485$. Magnification 210 ×.

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current to around 4 $\mu$A. Protein separation occurs thus in a polyacrylamide gel of 1.2 $\mu$l with a diameter of 200 $\mu$. The run takes around 10 min.

Certain factors have to be considered as possible sources of error in micro-disc electrophoresis as in any other type of electrophoresis using supporting media. It is of utmost importance to avoid electroendosmosis in microelectrophoresis. It can always be assumed that a layer of fluid exists between the outer part of the gel and the wall of the capillary in which molecular streaming can occur. HJERTÉN[12] has found that if the glass wall is coated with a methyl cellulose solution with a viscosity of 100 centipoises, electroendosmosis is completely avoided. Therefore, the capillaries used in our procedure have been coated with a thin layer of methyl cellulose (Methocel, Dow Chem. Co., Midland, Mich.).

Another factor of importance is the temperature gradient from the center of the gel to the outside. The temperature in the middle of the gel must not exceed 40°. Any excessive cooling of the outside of the capillary relative to the inner part of the gel should be avoided[13]. If not, the excessive gradient will produce a bending of the fractions in the direction of the run. In the examples given in this paper (see Figs. 4 and 5) it can be seen that the stained protein discs are evenly horizontal. In polyacrylamide gels of larger diameter, 5 to 10 mm, GANDINI AND DRAVID[13] have pointed out that the glass wall of the tubes should increase when the maximally favorable current is determined in relation to the thermal conductivity of the glass and the external cooling system. This is less critical in micro-electrophoresis since the thermal capacity of the thin capillaries including the gel is small and an equilibrium easily obtained as is demonstrated by our results.

For the 400 $\mu$ diameter gels, the corresponding details are: 60 V for the first 20 min under which period the protein is concentrated in the upper 5 % gel and the current falls continuously from around 20 $\mu$A to 5 $\mu$A. At the stage of "steady stacking", 13 mm of the upper part of the capillary is cut. The separation of protein then proceeds on a 3 $\mu$l gel with a diameter of 400 $\mu$.

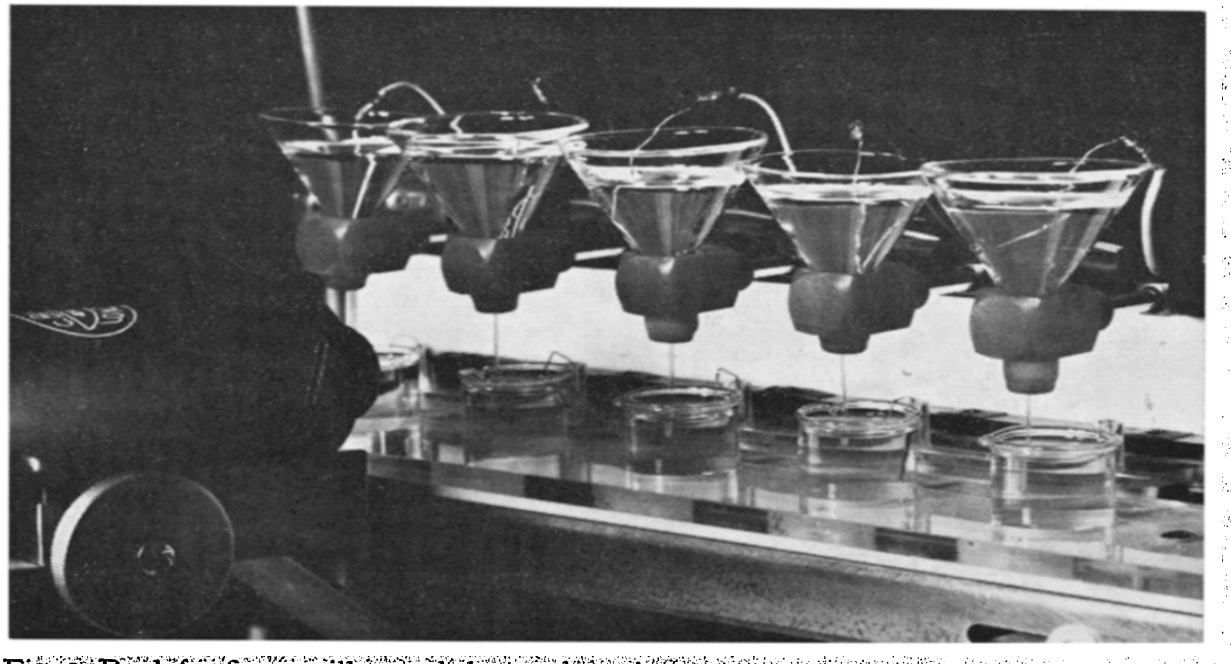

Fig. 2. Rack for five capillary gel electrophoresis.

After separation, the gel is removed by inserting a tightly fitting wire in the anode end of the capillary. The gel is extruded into 80 % ethanol. For staining, 0.5 % amido black in 7.5 % acetic acid is used for 5 min.

The apparatus for micro-separation consists of a rack for five capillary runs (Fig. 2). The jars for electrode buffer are placed on a vertically movable stand. The capillary holders are adjustable.

Fig. 3 gives a schematic outline of the procedure used and Figs. 4 and 5 show photographs of the 200 $\mu$ and 400 $\mu$ diameter polyacrylamide gels on which $10^{-7}$ g of brain protein have been separated for 25 min and stained by amido black.

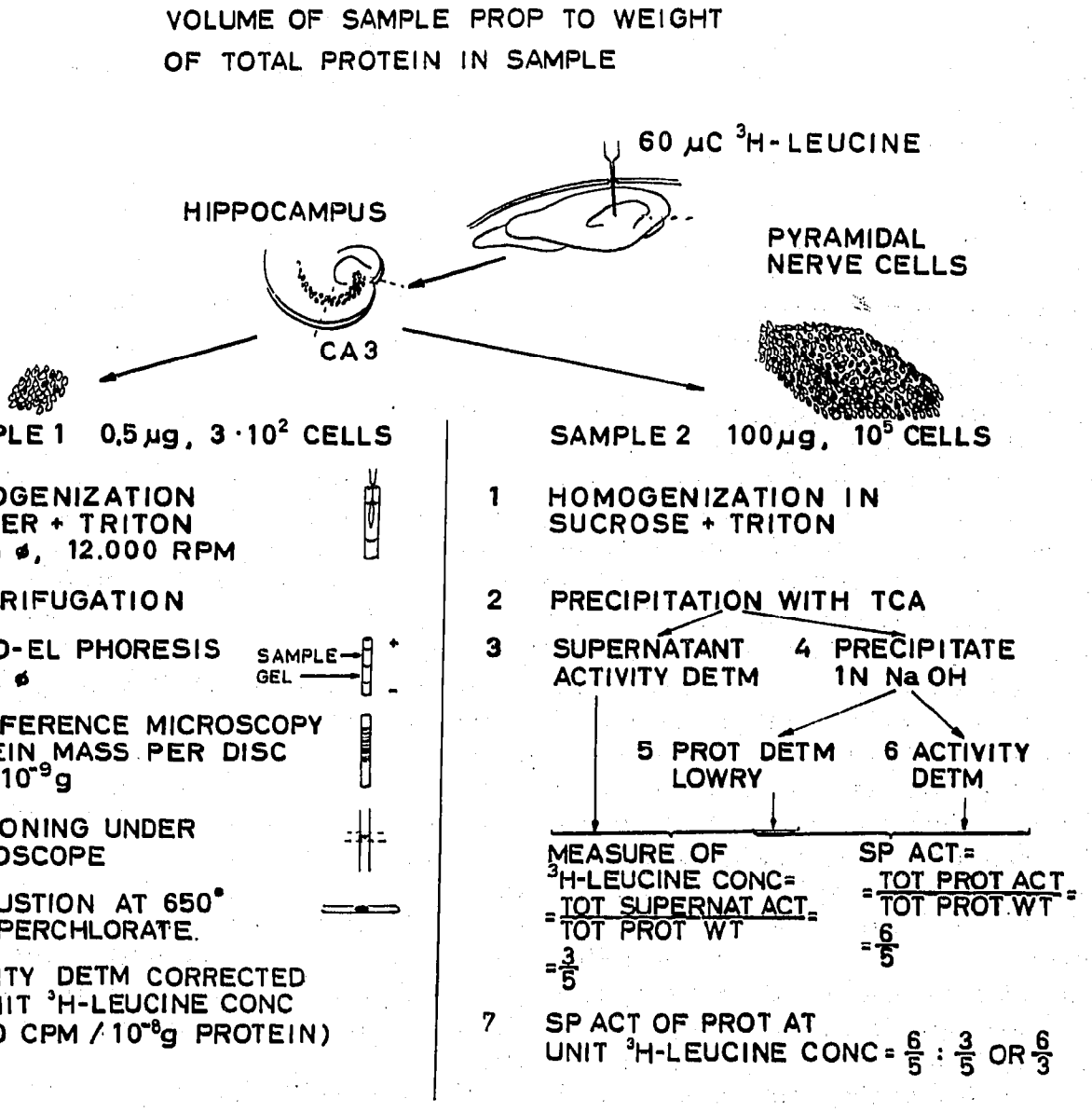


Fig. 3. Outline of procedure.

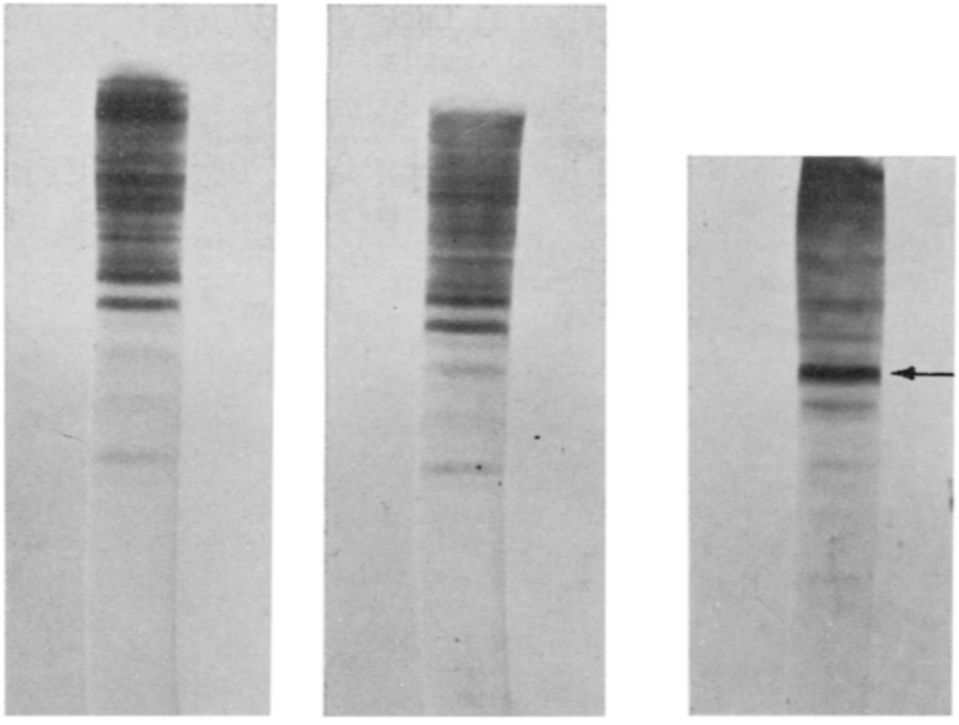

Fig. 4. Brain protein separation pattern on micro-polyacrylamide gels, 200 $\mu$ diameter, stained with amido-black. Magnification 25 ×.

Fig. 5. Brain protein separation pattern on micro-polyacrylamide gels, 400 $\mu$ diameter, stained with amido-black. Albumin added, fraction at arrow. Magnification 25 ×.

## RADIOMETRIC DETERMINATION OF THE PROTEIN FRACTIONS

The following description concerns the determination of the radioactivity in protein micro-fractions which have incorporated $^3$H-labeled amino acids. A method for determination of specific radioactivities of $^3$H-purines and $^3$H-pyrimidines has been described by KOENIG AND BRATTGÅRD[14]. The principle is to measure tritium radioactivity after the tritium has been converted to gas. By this means, the factor of self-absorption is removed or greatly reduced. In the protein analysis, we have followed the same principle and used the method with some modifications.

The protein fractions in the micro-gels are cut out under a stereomicroscope. By using a strictly standardized procedure, the various fractions could be localized by taking the fifth, stained gel as a reference. A small correction has to be made for the 10 % linear swelling which this gel undergoes in the acetic acid stain mixture. The cut-out gel segment containing the protein, 50 to 500 $\mu$ thick, is inserted into a Supramax glass capillary, i.d. 600 $\mu$ and 1.5 cm in length, together with powdered $KClO_4$ and Zn particles. The capillary is closed and heated at 650° for 45 min. This ensures oxidation to water and reduction to gas. The capillary is then introduced in a specially constructed counting tube provided with a mechanism for crushing the capillary, and letting the $^3$H gas escape into the carrier gas of the tube system which is a 99 % helium–1 % isobutane gas mixture.

The counting tube used by us is a Philips tube PW 4340, the mylar window of which has been removed. The radioactivity is counted with an anticoincidence probe

(Philips PW 4149) which has been adapted to a proportional spectrometer, Baird Atomic, model 563. The results are recorded by a Baird Atomic Printer 620. The efficiency of this combustion method was determined in the following way. One mm pieces of 440 $\mu$ diameter polyacrylamide gels with a $^3$H-activity of 39.6 c.p.m. per test piece were combusted and counted as described. Thirty determinations were performed and the result was 15 $\pm$ 1.5 c.p.m. per sample. The efficiency of the method was thus 40 %.

## THE INTERFERENCE MICROSCOPE TECHNIQUE

The calculation of the specific activity of the protein fractions after the micro-gel separation requires a determination of the amount of protein in the single discs (around $10^{-8}$ g). A direct determination of the weight of a protein fraction after its separation from the gel does not seem to be possible since the separation cannot be made quantitatively. The problem is therefore to determine exceedingly small amounts of protein in presence of large amounts of gel material. We have found that a possible way to solve the problem is to use an interference microscope technique. For this purpose a Leitz interference microscope was used ("Leitz Interferenz-Mikroskop für Durchlicht").

The objects to be investigated are thus protein discs in a micro-polyacrylamide gel with circular cross section (diameter around 200 $\mu$) or with a form of the cross section which has been more or less deformed. The refractive index of the gel material has been measured interferometrically and found to be 1.485 $\pm$ 0.005. The refractive index of proteins lies around 1.59. The micro-gel is embedded in a mixture of glycerol and benzyl alcohol with a refractive index of 1.485. This mixture penetrates the gel completely which can easily be seen from the image of the gel in the interference microscope. The embedded gel is placed at right angles to the interference fringes. If the refractive index of the embedding medium is exactly the same as that of the gel, the interference pattern in the microscope field is not influenced by the gel. However, generally there is a small difference in refractive index and the fringes within the gel are therefore slightly displaced (Fig. 6). This displacement (optical path difference of the gel relative to the embedding medium) is a function of the thickness of the gel and the (small) difference in refractive index between gel and the surrounding medium. Within the protein discs, the displacement of the fringes is the sum of the displacement due to the gel and that of the protein, the latter being, of course, in its turn a function of the amount of protein and the difference in refractive index between the protein and the embedding medium. Thus, the optical path difference for a point within a protein disc is $F = F_p + F_g$, where $F_p$ is the optical path difference due to the presence of protein and $F_g$ that of the gel. Further, as is well known

$$F_p = d_p(n_p - n_m) \text{ and}$$

$$F_g = d_g(n_g - n_m),$$

where $d_p$ is the effective thickness (*i.e.* the compressed, pore-free thickness) of the protein, $n_p$ the refractive index of the protein, and $d_g$ and $n_g$ the equivalent symbols

for the gel. The refractive index of the embedding medium is $n_m$. Thus, the general expression for the displacement (optical path difference) within a protein disc is

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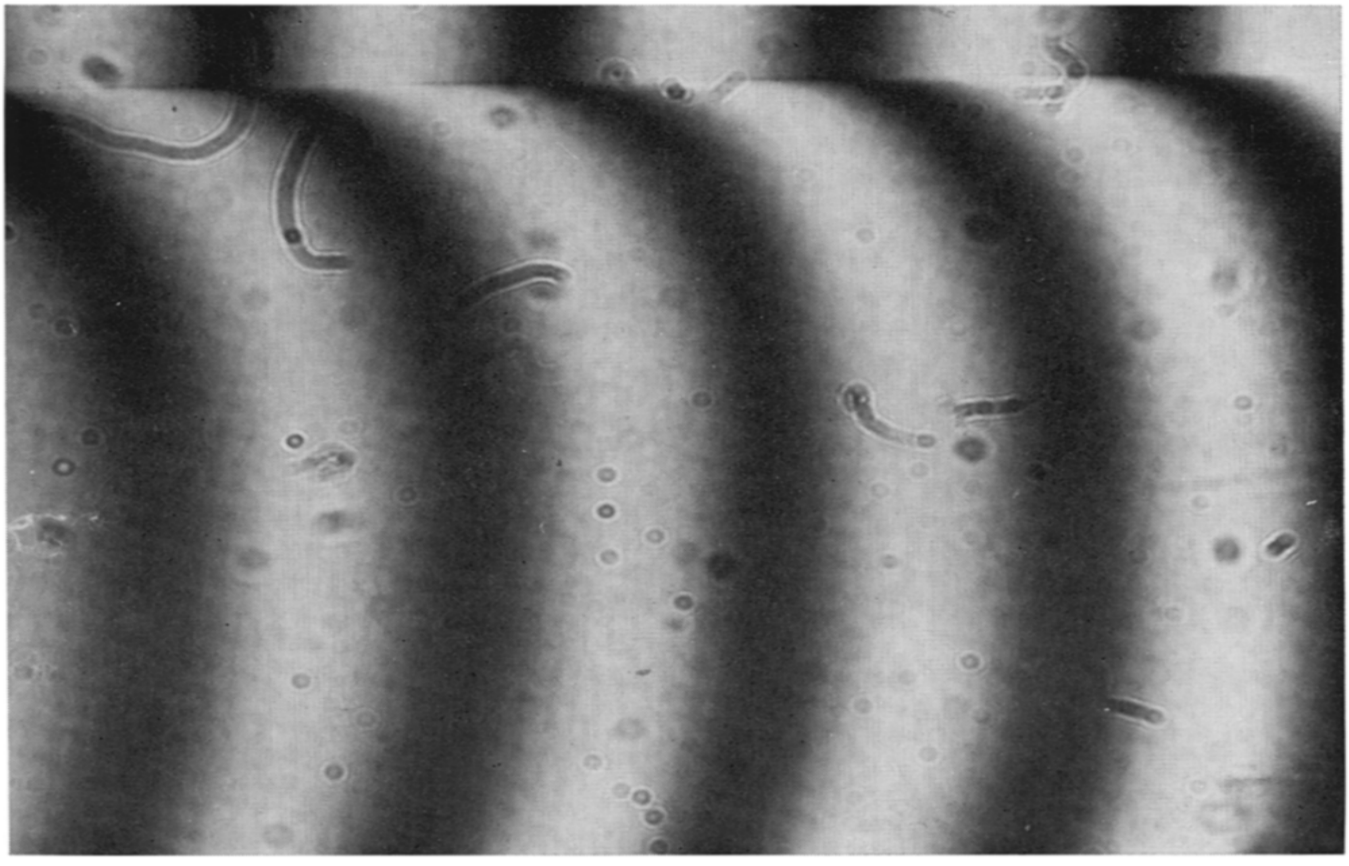

Fig. 6. Interference micrographs of a 200 $\mu$ diameter gel embedded in benzyl alcohol–glycerol, $n = 1.485$. Magnification 210 ×.

the amount of protein is so small, the displacement of the fringes within the disc relative to that of the fringes of the gel is also relatively small and not easy to estimate visually. The following procedure is therefore used. The density of the photographic plate with the interference image of the gel with the protein disc is first recorded along the line A—B in the middle of the protein free gel. This record is made on transparent paper. The second record is made along the same line from B to C where the protein disc is situated. When placing the first record (on transparent paper) above the second, it can easily be seen where the fringes are displaced owing to the presence of protein and thus the protein disc is located. It is also easy to measure the optical path difference within different parts of the disc. ($D_1$, $D_2$ and $D_3$ in Fig. 7).

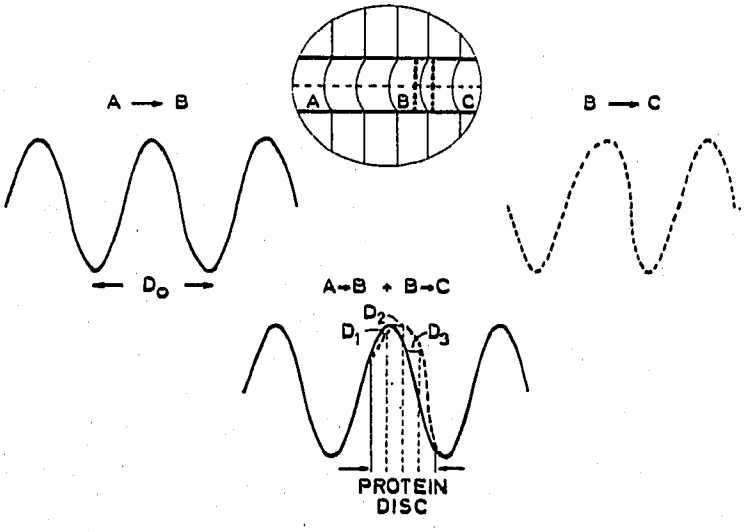


Fig. 7. Principles of micro-interferometric determination of protein amount in separated fraction.

The optical path differences are in this case $D_1/D_0 \cdot \lambda$, $D_2/D_0 \cdot \lambda$ and $D_3/D_0 \cdot \lambda$, where $D_0$ is the length of separation between the fringes on the record and $\lambda$ the wavelength used. If the thickness of the disc along the direction of the gel is constant and uniformly filled with protein, and if the gel is circular, the calculation of the protein content in the disc from a record along the A–B–C line is simple. However, for a lens form of the disc and/or a non circular cross section of the gel and with a non-uniform distribution of protein within the disc, several records (usually three or four) along the direction of the gel have to be made. We then obtain a distribution of $d_p$ values within a disc. Through simple graphic integration methods the amount of protein within a disc can be calculated.

The accuracy of the method is mainly dependent upon the accuracy of the displacement measurement. It would seem reasonable (with large fringe separation) that the error should not exceed 1/50 $\lambda$ and under favourable conditions 1/100 $\lambda$. In our measurements, the optical path differences are in the region of 1/10–1/4 wavelength which means that the error would vary in the region of 8–20 % or under favourable conditions 4–10 %.

Fig. 8 shows part of two records of the interference fringes in the gel outside and within a protein disc, situated above each other. The displacement of the fringes owing to the presence of a protein disc in the gel is easily seen.

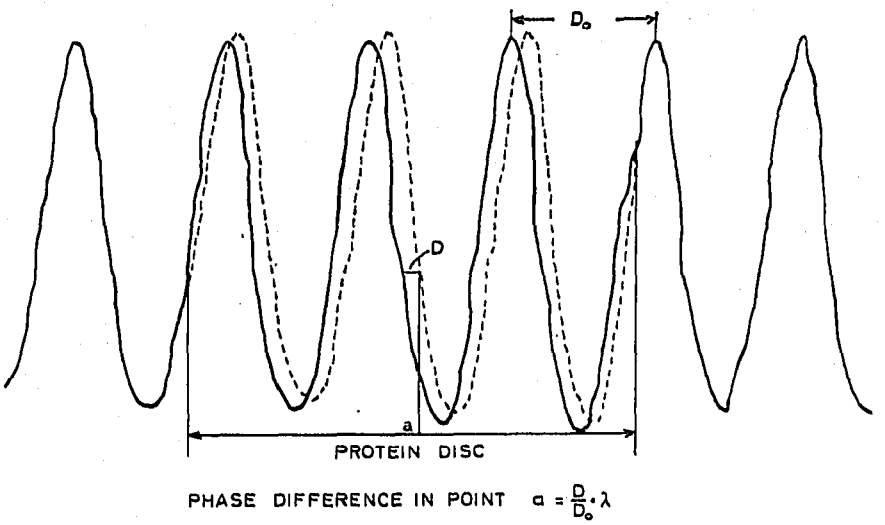


Fig. 8. Densitometric record of interference fringe displacement in protein disc.

The following practical example shows a simple case when 0.02 $\mu$g of albumin was analysed. The pattern consisted of two bands, one very strong, the other very weak. The diameter of the gel with circular cross section was 230 $\mu$. The strong disc was plane parallel with a thickness of 110 $\mu$. The separation of the recorded fringes was 79 mm. The displacement within the disc at the middle of the gel was 14 mm. The wavelength used was 546 nm or 0.546 $\mu$. The optical path difference of the protein (through the gel diameter) is thus $14/79 \cdot 0.546\ \mu = 0.097\ \mu$. The refractive index of albumin is around 1.60 and that of the embedding medium is 1.485. Thus $0.097 = d_p$ (1.60—1.485) from which we obtain $d_p = 0.097/0.115 = 0.84\ \mu$. This means that every $\mu^3$ in the disc contains $0.84/230\ \mu^3$ of protein. The volume of the disc is $\pi \cdot 115^2 \cdot 110\ \mu^3$. Thus the disc contains $\pi \cdot 115 \cdot 110 \cdot 0.84/230\ \mu^3$ of protein. If the density of the protein is set at 1.3, the amount of protein is

$$\pi \cdot 115^2 \cdot 110 \cdot \frac{0.84}{230} \cdot 1.3 \cdot 10^{-12}\text{g} \simeq 2.2 \cdot 10^{-8}\ \text{g}.$$

The smallest amount of protein which can be measured in a disc by this method (gel diameter ~ 200 $\mu$) can be estimated as follows:

Assume $D_0 = 100$ nm and $D = 2$ nm. Further $n_p = 1.60$ and $n_m = 1.485$. The thickness of the protein disc = 10 $\mu$ and the density of protein ~ 1.3.
This gives

$$\frac{2}{100} \cdot 0.546 = d_p \cdot 0.115. \quad d_p = \frac{2}{100} \cdot \frac{0.546}{0.115}$$

The weight of the protein in the band is thus

$$\pi \cdot 100^2 \cdot 10 \cdot \frac{2 \cdot 0.546}{100 \cdot 0.115 \cdot 200} \cdot 1.3 \cdot 10^{-12}\text{g} = 2 \cdot 10^{-10}\text{g}.$$

The error will be around 50 % for this extremely small amount of protein.

## CORRECTION FOR VARIATION IN PRECURSOR CONCENTRATION

When comparing the specific activity of the protein bands (*i.e.* the incorporation of labeled amino acids) or that of unseparated proteins from corresponding regions in two brain halves or from identical brain areas of different animals, it should be observed that the *concentration* of available labeled amino acids may show great variations. These variations are a function of different circulation conditions, dilution through cerebrospinal fluid, etc., all factors which are difficult or impossible to control. It is, therefore, necessary to obtain a measure of the concentration of the labeled amino acids in the investigated brain region in order to be able to compare the incorporation of the labeled amino acids in the two brain hemispheres or in the brain of different animals.

It is obvious that higher concentrations of amino acids should result in a higher incorporation of them, up to a certain saturation limit. In order to compare quantitatively the incorporation of labeled amino acids (expressed, for instance, as specific activity of unseparated and fractionated proteins from the brain regions investigated) it is necessary to correct for the influence of the variations in the labeled amino acid concentration. This means that it is necessary to have information concerning the (mathematical) relation between the specific activity of the proteins and the concentration of the labeled amino acid in the cells from which the proteins are isolated. If this relation is known, the specific activity of the protein fractions in a micro-gel can be referred to the same amino acid concentration and a quantitative comparison of the incorporation of precursors between different brain regions is possible.

In order to investigate whether a linear relationship exists between the protein specific activity and the free amino acid concentration, and the possible range of such a relation, the following experiments were made. See scheme in Fig. 3.

$^3$H-Leucine was injected bilaterally in varying amounts (5–60 $\mu$l, 1 $\mu$C/$\mu$l) into the lateral ventricles of a total of 20 rats, each weighing 175–200 g. The animals were killed 1.5 h after the injection. The brain was rapidly removed, and the two CA3-regions of the hippocampus (selected as a brain region of interest in connection with a behavioural experiment) were immediately dissected out and processed as outlined in the right half of the scheme in Fig. 3. About 100 $\mu$g of pyramidal neurons from the sample was homogenized in 0.5 % Triton X-100. The solution was precipitated with TCA. The precipitate was washed and its total radioactivity determined (c.p.m.). It was then dissolved in 1 *M* NaOH and the amount of protein ($\mu$g) in the solution was determined according to LOWRY's method[15]. In the supernatant, the total radioactivity of $^3$H-leucine was determined (c.p.m.). From these two latter determinations, a measure of the concentration of $^3$H-leucine in the cells investigated is obtained as the quotient between the total $^3$H-leucine activity and the amount of protein in the sample. The pre-requisite for this procedure is that the amount of protein in the sample can be used as a measure of the sample volume. It seems reasonable to assume that for identical regions in the two hemispheres or in the brains from different animals, the amount of protein per unit volume of that region should vary within narrow limits. It is therefore obvious that the incorporation of amino acids can be compared only for identical brain regions in the same or in different animals. Thus, the protein content of different brain regions with different cell structures cannot be compared with respect to the volume of the different regions. It should further be

observed that this method cannot be used for a comparison of the volume of identical brain regions from pathological and normal cases.

Fig. 9 shows the results. The specific activities of the unseparated proteins from the 100 $\mu$g samples of the CA3-region (c.p.m./$\mu$g protein) are plotted against the concentration of $^3$H-leucine (c.p.m. of the supernatant per $\mu$g protein in the sample).

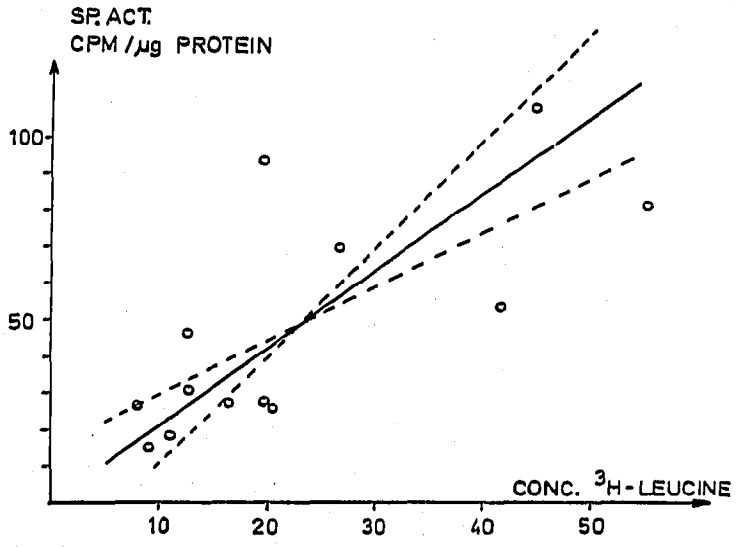


Fig. 9. Linear relation between protein specific activity and $^3$H-leucine activity.

A regression analysis shows that a linear relationship exists between the variables with a high degree of significance ($p$ = 0.01—0.001). The correlation coefficient has the same degree of significance. With a high degree of significance the experiment has thus shown that within the concentration region up to 50 units (as described above) the specific activity of the protein is directly proportional to the concentration of the $^3$H-leucine.

Since a linear relationship exists between specific activity and amino acid concentration, it can finally be stated that the specific activity of unseparated proteins from a 100 $\mu$g sample or of a protein band in a micro-gel can be compared with the equivalent activities of samples from the identical brain region of the other hemisphere in the same animal or from different animals simply by dividing the specific activity by the $^3$H-amino acid concentration. All values of the specific activity are thus compared at the same $^3$H-amino acid concentration, here called unit $^3$H-leucine concentration.

## APPLICATION

The following example of the application of the micro-procedure for protein analysis is given to illustrate its potentialities.

Rats were used in an experiment to induce a transfer of handedness in the retrieval of food and to study the effect in isolated nerve cells of a control area of the cortex. No surgical measure was undertaken to induce the animals to switch from the preferred paw to the other paw in taking small food pills from far down a narrow

glass tube. A wall was simply arranged close to and parallel to this tube. Since the animal cannot reach over cross-wise with the preferred paw, it tries to use the other paw and eventually learns. Two training periods per day were given. On the 4th–5th day the rats were still on the rising part of the performance curve. Nerve cells from that period were isolated from the so-called CA3-region in the hippocampus which contains medium-sized pyramidal nerve cells. One hour and a half before the sampling of material, 60 × 2 $\mu$l with 1 $\mu$C/$\mu$l of a $^3$H-leucine solution was injected bilaterally in the lateral ventricles. Nerve cell bodies from the CA3 area of the hippocampus were sampled, homogenized and protein extracted with a salt solution containing Triton X-100 to solubilize the proteins, as described above under the Correction procedure. In the separation pattern of the proteins on the micro-gel, there are two prominent fractions on the acid half of the gel, designated 4 and 5 (Fig. 4).

The amount of protein of these two bands from the left and the right side were determined by the interferometric procedure. On the right side, corresponding to the learning paw, the protein amount was $2.08 \pm 0.15 \cdot 10^{-8}$ g and on the left side $1.96 \pm 0.15 \cdot 10^{-8}$ g. These paired values were obtained from the same animals. Protein samples from control rats were also separated; the protein amount of fractions 4–5 in this latter case was $2.02 \pm 0.15 \cdot 10^{-8}$ g.

The radioactivities of the samples after combustion were as shown in Table I.

TABLE I

TOTAL RADIOACTIVITY (C.P.M.) OF PROTEIN FRACTIONS 4 AND 5

| *Hippocampus, experimental rats* | | *Hippocampus, control rats, both left and right sides* |
|---|---|---|
| *Right side* | *Left side* | |
| 11.86 ± 1.53 | 14.31 ± 1.46 | 16.40 ± 1.46 |

Since the amount of proteins/sample is the same in the three cases, the total radioactivity of the fractions can be used as a measure of the specific activity. In order to be able to compare the specific activities of the different micro-fractions of proteins at the same concentration of $^3$H-leucine in the small, defined CA3 region, the correction was performed as described above, *i.e.* each value for the sum of the specific activities of the 4th and 5th band in the micro-gel was divided by the value of the $^3$H-leucine concentration in the sample from which the micro-gel sample was taken. This quotient is denoted by $I_p$ and can be used for comparison of the incorporation of $^3$H-amino acids into identical brain regions under different neural functions. After correction of the specific activity values for the variation in the $^3$H-leucine concentration, the values shown in Table II are obtained.

Furthermore, it can be mentioned that the average values of the $^3$H-leucine concentration in the three cases were 10.7 ± 1.11, 15.3 ± 2.32 and 30.4 ± 2.80, respectively. Thus, when the specific activities of the protein fractions 4–5 were corrected for the concentration of $^3$H-leucine in the CA3-region, it could be concluded that the specific activities of the nerve cell protein of the experimental rats were significantly higher than those of the control rats.

This is an example where corrected protein values have been determined both

TABLE II

CORRECTED TOTAL RADIOACTIVITY (*Ip*) OF PROTEIN FRACTIONS 4-5 (*cf.* TEXT)

| | *Hippocampus, experimental rats* | | *Hippocampus, control rats, both right and left sides* |
|---|---|---|---|
| | *Right side* | *Left side* | |
| $I_p$ | 1.20 ± 0.15 | 0.97 ± 0.08 | 0.51 ± 0.04 |
| No. of animals | 9 | 9 | 4 |
| No. of microgels | 17 | 20 | 16 |

from cells of the two halves of the same brain, as well as from cells from different animals.

## APPENDIX

*Polymerization of the lower gel*

*Stock A*. pH 8.8: 860 mg, Tris (The British Drug Houses, specially purified) + 0.063 ml N,N,N′,N′-tetramethylethylenediamine + 3.6 *N* $H_2SO_4$ + $H_2O$ to 10 ml and a pH of 8.8.

*Stock B*. pH 6.7: 2.85 g Tris + 1 *M* $H_3PO_4$ to pH 6.7 and $H_2O$ to 50 ml.

*Stock C*. 20 g recrystallized acrylamide (Eastman Organic Chemicals) + 200 mg N,N′-methylenebisacrylamide + 3.75 mg $K_3Fe(CN)_6$ + $H_2O$ to 37.5 ml.

*Stock D*. 70 mg $(NH_4)_2S_2O_8$ + 25 ml 2 % Triton X-100 + $H_2O$ to 50 ml.

*Stock E*. 5.98 g Tris + 0.46 ml N,N,N′,N′-tetramethylethylenediamine + 1 *M* $H_3PO_4$ + $H_2O$ to 100 ml and a pH of 6.7.

*Stock F*. 200 mg $(NH_4)_2S_2O_8$ + 5 ml 2 % Triton X-100 + $H_2O$ to 10 ml.

*Electrode buffer*. pH 8.5: 3.0 g Tris + 14.4 g glycine + $H_2O$ to 500 ml. Fluorescein: one drop of a saturated solution.

*Protein dye*. Amido black 0.5 % in 7.5 % acetic acid.

*Preparation*. One ml of (0.5 ml Stock A + 1.5 ml Stock C) + 1.0 ml Stock D, gives a 20 % solution. To this is added 100 mg acrylamide + 10 mg hydantoin in order to get a final 25 % polyacrylamide gel.

*For homogenization*. 20 μmole sodium thioglycolate is added to the sample in a 0.25 *M* sucrose + 0.5 % Triton X-100 solution, buffered by Stock B to pH 6.7.

*Polymerization of the upper gel*

One ml of (0.5 ml Stock E + 1.5 ml Stock C) + 1 ml $H_2O$. One ml of this solution is added to 1 ml Stock E. One ml of this solution is added to 0.8 ml $H_2O$ + 0.2 ml Stock F. The final concentration of this upper gel will be 5 %.

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## REFERENCES


1 H. HYDÉN, K. BJURSTAM AND B. MCEWEN, *Anal. Biochem.*, 17 (1966) 1.
2 H. HYDÉN, *Nature*, 184 (1959) 433.
3 H. HYDÉN AND S. LARSSON, *J. Neurochem.*, 1 (1956) 134.
4 H. HYDÉN AND B. ROSENGREN, *Biochim. Biophys. Acta*, 60 (1962) 638.
5 B. H. O. ROSENGREN, *Acta Radiol. Suppl.*, 178 (1959).
6 H. HYDÈN AND P. LANGE, *Acta Physiol., Scand.*, 64 (1965) 6.
7 H. HILLMAN AND H. HYDÉN, *Histochemie*, 4 (1965) 446.
8 H. HILLMAN AND H. HYDÉN, *J. Physiol.*, 177 (1965) 398.
9 H. HANSSON AND P. SOURANDER, *Z. Zellforsch. Mikroskop. Anat. Abt. Histochem.*, 62 (1964) 26.
10 D. EICHNER, *Experientia*, 22 (1966) 620.
11 J. M. BREWER, *Science*, 156 (1967) 256.
12 S. HJERTÉN, *Thesis*, Uppsala, 1967; *Chromatog. Rev.*, 9 (1967) 122.
13 S. GANDINI AND A. DRAVID, *Proc. Intern. Meet. Soc. Neurochem., 1st, Strasbourg 1967*, p. 77.
14 E. KOENIG AND S.-O. BRATTGÅRD, *Anal. Biochem.*, 6 (1963) 424.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
16 U. E. LOENING, *Biochem. J.*, 102 (1967) 251.
17 V. NEUHOFF, *Arzneimittel-Forsch.*, 18 (1968) 35.