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Agar Gel Electrophoresis in Neurology

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AGAR GEL ELECTROPHORESIS IN NEUROLOGY

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Antwerp, June 1964 A. LOWENTHAL

CONTENTS

Chapter 1

INTRODUCTION

The need for a qualitative test for the proteins of the cerebrospinal fluid (CSF) had imposed itself ever since the introduction of the lumbar puncture. The proposed techniques were first of all those of colloidal precipitation of proteins (colloidal gold, colloidal benzoin, collargol). The significance of the results has always remained in dispute and their physicochemical interpretation difficult, in spite of careful and repeated studies, particularly during the last few years. These methods, however, had their usefulness. They made it possible to prove that in some cases unquestionable qualitative differences exist between the proteins of the CSF among patients afflicted with various neurological syndromes. Among these methods must be mentioned the technique for the determination of total proteins and the definition of the albumin/globulin ratio, described by Kafka. The modifications observed in some cases have been discussed. Ever since 1946 Kafka clearly laid down the basis of a problem which is to become essential: what could be the origin of the γ-globulins that can be found in excess in the CSF, the total protein content of which remains normal?

Chemical methods have also been used to determine the chemical composition of the proteins of CSF. Stary (1931) and Yde (1934–1937) tried to separate the different fractions by salting out. Yde was thus able to show increases in globulins in generalized paralysis and cerebral tumors. Other authors (Ujsaghi 1941, Abelin 1943, Lehmann 1945, Epstein 1947) have shown that unquestionable qualitative differences exist between different fluids. The above authors could, however, not define these in a more precise way. In fact, all these methods have been applied to the proteins of the CSF by comparison with serum proteins. Certain authors have also created and applied more specific methods and so, finally, a very large number of colloidal reactions has been proposed. These were to confirm that the proteins of the CSF could present qualitative differences, without apparent quantitative modifications. In spite of many attempts, these qualitative differences could never be defined in a more precise way. Kafka was able to underscore that a crisis has resulted from the fact that, although it has been proved that qualitative differences

could appear in the proteins of the CSF, the techniques used for their distinction remained empirical and ill defined. In 1946 Kafka was in a position to say that although the study of the proteins of the CSF was only at its beginning, it seemed to be most promising. He was able to add that he knew of no method that could be applied with success to the solution of this problem.

The opinion of Kafka was too severe. A few electrophoretic examinations of the CSF had already been carried out at this time. These initial studies, however fragmentary, gave extremely important indications. It is surprising that the many ulterior studies devoted to the electrophoresis of the proteins of the CSF, have not contributed many new elements. The method of Tiselius, boundary electrophoresis, made it possible to show, ever since 1939 (Hesselvik), that the proteins of the CSF were constituted of different fractions. It has been possible to study two CSF's. Kabat (1942) believed to be able to show by the same method that in myeloma there is an increase of γ-globulins in the serum and in the CSF. He was also able to show, maybe for the first time, the increase of the γ-globulins in multiple sclerosis. In 1942 Kabat, working still with the same technique, compared the proteins of the CSF to the proteins of serum. He could not find γ-globulins, nor fibrinogen, in the normal CSF. On the other hand, he determined that the γ-globulins are relatively increased in syphilis and in multiple sclerosis. Already at this time Kabat raised the question if some of the γ-globulins thus observed are not formed in the central nervous system. The same year, in a separate study, Kabat observed that in myeloma one finds parallel modifications of serum proteins and of proteins of body fluids. About the same time and independently of Kabat and his co-workers, Scheid *et al.* (1944) seek to explain the simultaneous modifications of colloidal curves and of electrophoretic fractions of the CSF. For Scheid, the proteins of CSF are similar to serum proteins in Guillain-Barré disease, poliomyelitis, meningitides and cerebral tumors. This similarity would be due to modifications of permeability of the blood–brain barrier. Booij (1949, 1950, 1952) also devotes a series of studies to the electrophoretic composition of the proteins of CSF. He uses boundary electrophoresis with a special cell, permitting the use of small volumes of CSF. Booij could not find γ-globulins in normal CSF. He observes a modification of β-globulins in inflammatory syndromes and he assumes that these proteins thus increased are formed within the central nervous system. He also observes an increase of γ-globulins in meningitides and in syphilis. Booij also insists on the fact that one always finds albumin in CSF. The significance of electropho-

retic studies of CSF proteins has been stressed by Trincao (1961). He insisted upon the fact that the CSF proteinogram is slower to return to the normal than the other biochemical characteristics of this fluid.

By reviewing these studies predating the introduction of paper electrophoresis one is surprised by the fact that already at that time, the same conclusions have been drawn as today, and that most of the presently unsolved problems have already been laid down. These studies already contain the nucleus of all the problems confronting the contemporary investigator.

(1) It was admitted from the start that there existed an identity between *serum proteins and CSF proteins*. This hypothesis is still frequently defended. Authors using immunoelectrophoresis (Burtin 1960, Frick 1957) have stated recently that the proteins of CSF are identical to the serum proteins, and if qualitative differences exist between the proteins of CSF and of serum, these could not be demonstrated. The conclusions of these authors using immunoelectrophoresis can not be accepted, however, without reservations. If so, one would have to state as a paradox that there is practically no β_2-globulin in the CSF, even though there exists manifestly a fraction which follows immediately the β_1-globulin and which is very characteristic of the CSF. Then it is indispensable to define this fraction with more precision. This might even bring us to say that in leucoencephalitis immunoelectrophoresis shows that the proteins of CSF are normally distributed, which is clearly wrong, as can be shown by paper as well as agar gel electrophoresis. Hence there is a problem which must certainly be discussed, and discussed in a very rigorous manner: the problem of relations between serum proteins and proteins of CSF.

(2) A second problem has also been raised early, as we already indicated: the problem of the possible *origin of abnormal proteins of the CSF*. More particularly, the origin of excess γ-globulin in CSF in certain affections, such as multiple sclerosis, was in question. This same question will come up later in relation with the γ-globulins of subacute sclerosing leucoencephalitis. It could be asked if these γ-globulins are not formed in the central nervous system.

(3) These initial studies underscore remarkably the essential *technical difficulty* which is to be met. The studies of Kabat, of Scheid, of Booij could not be further developed, because at the time there was no good *method for the concentration of CSF*. Considerable quantities (100–150 ml) of CSF must be used. These methods could therefore not be used in the clinic. They were strictly laboratory methods. The problem presented by the dilution factor of

the proteins of CSF has been solved later by the introduction of good methods of concentration.

The use of these methods will again bring up a new problem: to determine *how much the procedures employed can modify the composition of the CSF.* This problem will not be discussed here, we shall mention it later. It is certain that the concentration methods have an influence upon the proteins of CSF, it is also certain that these methods can modify the electrophoretic distribution of the proteins. The discussion of these methods, the search for the most appropriate methods will retard considerably the development of our knowledge of the proteins of CSF. This difficulty will, however, be overcome. At this moment the *interpretation of the results* will constitute a new problem. This problem has two aspects: first, how to express the concentration of each of the fractions demonstrated? second, how to name these different fractions?

In order to be able to express the absolute concentration of each fraction, it would be necessary to dispose of a good practical method for the calculation of total proteins of CSF. We shall not return to this problem in detail. Steger has done so in 1954 and has shown that, in fact, we do not have a good method of determination of total proteins for the CSF. We can only express our results in relative concentrations. This certainly poses problems with the mathematical discussion of the values obtained.

The naming of the fractions has always been made in comparison with serum. This method is insufficient, as we shall show later. The use of relative mobilities in an electrophoretic field, to be discussed later, will bring considerable precisions in this field.

Reviewing the studies predating the introduction of paper electrophoresis, it can be noted with some surprise that certain facts were learned and that certain problems were posed in terms similar to those used today; this in spite of a considerable number of publications devoted to the electrophoresis of the proteins of CSF.

It may well seem that from the moment that paper electrophoresis is to be applied to the proteins of CSF, it can already be known that there will be differences between the electropherograms of CSF and of serum. It can also be known that there will be a rise in γ-globulins in multiple sclerosis, syphilis and diverse types of meningitides. The question of the origin of these γ-globulins arose already before the introduction of paper electrophoresis and it can be asked if these are not formed within the meningeal space. The

problem of the origin of the proteins of CSF has already been discussed and required already at this stage a more precise definition.

However, if this seems clear to us now, *a posteriori*, one should not forget that what has been learned before the introduction of paper electrophoresis is still open to debate and that the problems thus presented are not very precise. Due to paper electrophoresis, the number of studies will grow in enormous proportions and more precise and undisputable answers will be given to some of the questions presented.

As already mentioned, the application of *paper electrophoresis* to the study of the proteins of CSF presented the preliminary problem of the concentration of fluids. This problem will have to be discussed at length. The use of paper electrophoresis makes possible the confirmation of the finding that in many neurological syndromes a relative increase in γ-globulins can be registered. Paper electrophoresis will yield some indications about the modifications of β-globulins, which some authors wanted to designate as specific proteins of the CSF. Finally, paper electrophoresis has permitted the presentation of a new problem: are there qualitative differences or only quantitative differences between the normal and pathological protein fractions of the CSF? Are there qualitative differences or only quantitative differences between the γ-globulins which appear in excess in multiple sclerosis, syphilis, various encephalitides, and the γ-globulins that can be shown in normal CSF or normal serum?

Curiously enough, beginning with the hypothesis that there exist qualitative differences between the proteins of CSF in various neurological syndromes, we have come to ask ourselves anew: are there qualitative differences between the γ-globulins of the CSF in various neurological syndromes? This certainly constitutes a forward step and also explains why paper electrophoresis can not be considered sufficient in all cases. This new conclusion, this new problem, justify the application of new electrophoretic techniques to the proteins of CSF. These are starch and agar gel zone electrophoresis, the study of enzyme activities of proteins (enzyme electrophoresis), and particularly the discussion of earlier results by the study of a larger number of cases.

The question which we shall attempt to answer here is whether the application of these new methods to the proteins of CSF has brought new indications, or whether it has made it possible to direct the discussions and to derive new conclusions.

The importance of the problems brought up by the use of paper electrophoresis in connection with γ-globulins is clearly stressed by the publications of the last few years. These frequently attempt to define the importance of quantitative modifications of these globulins. With this purpose, the most diverse and the most curious techniques are proposed: Papadopoulos (1959) tries to return to the study of γ-globulins by a chemical method and shows that the ratio total proteins/ γ-globulins is lowered in multiple sclerosis. This chemical method is discussed in turn by Pette (1958), who states that it has little value. If some authors attempt to complete electrophoretic results by chemical methods, others attempt to return to the position of 1952 by comparing again electrophoretic results and clinical data. Thus Cervos Navarro (1959) reviews completely the results of a very large number of cases and discusses them with reference to the clinical syndrome. Habeck does the same (1960) and tries to define the normal CSF. In a survey made with many laboratories, he tries to compare the methods used by various investigators and must admit that considerable divergences exist. Because of these divergences the interpretation of the pherograms is not always the same with all the authors.

This crisis of paper electrophoresis is manifested by the denial of its usefulness by some authors who propose new methods for the determination of proteins and of globulins. Rappaport (1959), Andersch (1960) etc., even renounce the use of paper electrophoresis. They try to define more precisely the proteins of the CSF with methods other than electrophoresis. On the other hand, a third group of authors will attempt to learn more about the proteins of CSF by comparison with serum proteins. Wender (1959) studies the modifications of serum proteins in neurological affections, in order to find the repercussion that they might have on the proteins of CSF. In the same manner, serum proteins are being studied in meningitis, chorea, the syndrome of Guillain-Barré, and diffuse sclerosis, in order to attempt to draw from these serum studies the conclusions concerning the chemical composition of CSF.

Electrophoresis has in fact brought new information, but this information remains difficult to interpret. After reading many publications, by attending meetings where the results obtained by different authors were discussed, we came to the conclusion that new methods of electrophoresis should be used for the study of the proteins of CSF, in order to attempt a more precise definition of the changes demonstrated by paper electrophoresis. This is

why we shall first discuss in as great detail as possible the results of paper electrophoresis and set out the conclusions obtainable with this method. We shall then discuss the contribution of new methods, agar gel electrophoresis and enzyme electrophoresis, to the knowledge of the proteins of CSF.

Our work will contain the following parts:

1. The application of the techniques in use to the CSF.
2. Paper electrophoresis.
3. Agar gel electrophoresis.
4. Serum proteins and the proteins of the CSF in neurological affections of animals.
5. Initial results obtained in the electrophoresis and enzyme-electrophoretic studies of the proteins of the nervous system.
6. Immunoelectrophoresis.

Chapter 2

TECHNIQUES

First we shall discuss the conditions of sampling, then the methods of concentration, of electrophoresis, of fixation and of demonstration of protein fractions.

After electrophoresis, there remains the question of interpretation of the electropherograms. The identification of demonstrated protein fractions, the quantitative interpretation of the results are so many questions which we shall attempt to answer. In this latter part we should discuss the results as a function of the age of the patients under study, and we shall compare the results obtained from the human CSF to the results obtained from various animals.

1. CONDITIONS OF SAMPLING

The CSF is most often sampled by the classical lumbar puncture. It is necessary to centrifuge after sampling, in order to eliminate the red blood cells as well as the leucocytes. The centrifugation is not very important for the proteinograms proper, but is extremely important for the enzymatic study. Our personal experience indicates that the CSF sampled under these conditions can be conserved at a temperature of 4° C or better between –20° C and –30° C for a long period of time, provided it is sterile. At 4° C a conservation of 8 to 15 days is possible, between –20° C and –30° C the conservation is practically unlimited. This permits the examination of CSF after shipments of several days. Before any electrophoretic examination the total protein content and the cell content of the examined fluid should be known. This has been done in all our cases.

2. CONCENTRATION OF THE CSF

Very different methods have been proposed for the concentration of the CSF. We must cite here those of Esser (1952), Bauer (1953), Steger (1953), Rossi (1953), Mies (1953), Schmidt (1955), de Risio (1957), and Barre (1959). The various techniques proposed include lyophilization, dialysis, precipita-

tion by acetone or by phytic acid, ultrafiltration under high pressure of air or nitrogen, ultrafiltration under vacuum (Bauer 1959). We shall not discuss these methods, we shall mention only a few of them, and eventually we shall present our personal experience. Dialysis seems of little interest to us. It is time consuming and often causes changes in the proteins. Acetone precipitation, described by Bücher (1952) and the simplified version of Roboz, has been used by us in the beginning. It certainly yields good results. The technique of Bücher is complicated and demands expensive equipment; the concentration is done in a cold chamber. The modification of Roboz (1954), which we have introduced independently from this author, is certainly much simpler, in fact so simple that a more extensive denaturation of proteins would be expected by its use. The proteins of CSF can be concentrated by acetone precipitation simply in tubes chilled by melting ice in the presence of NaCl. In spite of the simplicity of this method, the results are good. A partial loss of α-globulins is observed however with this method and in general with all acetone precipitation methods. This phenomenon has been described by several authors and has been recalled recently by Burtin (1960). The methods of lyophilization and of dialysis demand a complicated equipment and are fairly difficult to use. Furthermore the results obtained by dialysis are far from perfect, as we have already mentioned. A good method of concentration must be primarily a fast one. For this reason it seems that ultrafiltration is the best method, either under high pressure or by vacuum. Ultrafiltration under high pressure of air or nitrogen (10 atmospheres) seems to allow an easier collection of the concentrated fluid than ultrafiltration under vacuum as proposed by Mies. Our method of ultrafiltration could be criticized because of the necessity to replace the filters* at frequent intervals (one filter serves only twice) and to dispose of a well defined piece of equipment. It could be particularly criticized because recuperation of the proteins on the filters is not necessarily quantitative. Recently Colover (1961) has proposed at the Hamburg meeting on the proteins of the CSF a new method of concentration, based on an entirely different principle. This method consists of extraction of the water from the CSF through a semi-permeable membrane by the action of a hydrophylic substance (phytic acid). The concentration chamber proposed by Colover is extremely ingenious. The duration of the concentration (12 hours) seems however too long and risks to cause changes in the proteins. On the other hand, this method presents the great advantage that the proteins are not

* Filter Membran Gesellschaft, Göttingen, Germany.

collected on a filter, but at the bottom of a cell and that an almost quantitative recovery is possible. A smaller volume of CSF might therefore be sufficient.

3. ELECTROPHORESIS PROPER

Two methods of electrophoresis will be discussed here, because we have applied them to important series of the CSF. These are paper electrophoresis and agar gel electrophoresis. The latter constitutes a definite progress over the former.

Paper electrophoresis has become at present a routine method for serum and even CSF analysis. The CSF is concentrated and applied to the paper on the cathode side. The paper is placed in an electrophoretic chamber with its ends soaking in a buffer solution made of sodium veronal (pH 8.4). The pH of this buffer must be strictly controlled. The slightest modifications yield differing results (Yeoman 1959, Kunkel 1959). A potential is applied to the two terminals of the electrophoretic chamber. The different proteins will migrate until equilibrium is established. The ionic strength has only the slightest role here. After electrophoresis of about 15 h, the paper is dried, either at room temperature in the air, or in the incubator at 37° C. The drying can be done in the horizontal or vertical position. We believe that these different methods influence very little the results of the examination. The dried paper is then stained, either by amido black or by bromphenol blue, in order to color the protein, or by Sudan black for the lipoproteins, according to the technique of Swahn; or by the more complex method required by the Schiff reaction to demonstrate the glycoproteins. It seems that the method with amido black (amidoschwarz) might be the most favorable as it was demonstrated by Sunderman (1959). Other methods, for example the staining with azorubine (Balado 1959) show that the discussion is not yet completely settled and that the search for modifications and improvements of the technique used is still going on. The proteins can be either eluted or examined by densitometry for the quantitative evaluation of the separated proteins. Elution is a lengthy and fairly difficult method. It also destroys a document, the pherogram, that might be of value to preserve. It is certainly of greater interest to make the electrophoretic strip transparent by immersion in paraffine oil. The intensity of light absorption (optical density) can then be read from millimeter to millimeter and an extinction curve can be traced manually or this curve and the optical densities can be registered automatically. Once the division of the curve is obtained in fractions defined by their ex-