Studies on Sialic Acid (Neuraminic Acid) in Cerebrospinal Fluid

Part 4: Methodology of Determination by Diphenylamine Reaction

By

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INTRODUCTION

It has been recognized that sialic acid (neuraminic acid) exists widely in the mammalian body. The substance reacts characteristically to Ehrlich's p-dimethylaminobenzaldehyde reagent, Bial's orcinol reagent and Dische's diphenylamine reagent.

Sialic acid is closely related with the brain, as the name of neuraminic acid implies, and it is the characteristic component of the brain ganglioside.¹⁻⁷⁾ And there are many studies on it in the brain. The first report^{8,9)} of sialic acid in the cerebrospinal fluid (CSF) was given out in 1955. Since then, there have been some reports about it, in which Bial's reaction has been relied upon for estimation of the acid content. Since 1957, we¹⁰⁻¹⁶⁾ have been engaged in studies on sialic acid in human cerebrospinal fluid. In this paper are presented the results of investigation about the diphenylamine reaction as a method of colorimetric determination of sialic acid in CSF.

MATERIALS and EXPERIMENTS

Materials: Samples of CSF, containing less than 3 red blood cells per cubic millimeter, were obtained by lumbar puncture from human body. The samples of CSF contained from 0.15 to 0.45% of total protein by Kafka's method.

Experiments were made with samples of the whole CSF, the protein in CSF and the protein-free CSF. The details of the samples were as follows:

Sample 1: Whole CSF.

Sample 2: 2 ml of whole CSF plus 0.1~0.3 ml of saturated trichloroacetic acid.

Sample 3: Protein taken out¹³⁾ by precipitation from 10 ml CSF. 2 ml of redistilled water or 5 per cent trichloroacetic acid solution was added to the protein and mixed.

Sample 4: Niazi-State's fraction¹⁷⁾ (sialic acid) extracted from protein by the method described elsewhere.¹⁴⁾

Sample 5: Protein-free CSF. 0.5 ml of saturated trichloroacetic acid was added to 10 ml of CSF. The mixture was left standing overnight and centrifuged. The sample was the supernatant obtained thereby.

Sample 6: Concentrated protein-free CSF containing the trichloroacetic acid at the rate of about 5 per cent. 10ml of whole CSF was dried *in vacuo*. 2 ml of 5% trichloroacetic acid solution was added to the dried CSF. The mixture was stirred with a rod and left for 20 minutes; the protein was suspended. The tube was centrifuged. 5% trichloroacetic acid solution was added to the supernatant to the proper concentration.

Sample 7: Concentrated protein-free CSF. Protein in 10 ml of CSF was precipitated with 40 ml of absolute ethanol.¹³⁾ After centrifuging, the supernatant was dried *in vacuo*. Redistilled water was added to the dried protein-free CSF to the proper concentration.

Sample 8: Concentrated protein-free CSF, containing trichloroacetic acid at the rate of 5 per cent. Protein in 10 ml of CSF was precipitated with 40 ml of absolute ethanol. After centrifuging, the supernatant was dried *in vacuo*. 5% trichloroacetic acid solution was added to the dried protein-free CSF to the proper concentration.

Sample 9: Concentrated CSF containing trichloroacetic acid at the rate of 5 per cent. Whole CSF was dried *in vacuo*. A proper volume of 5 per cent trichloroacetic acid solution was added to the dried CSF. The protein in CSF was suspended.

Sample 10: Concentrated CSF. Whole CSF was dried *in vacuo*. A proper volume of redistilled water was added to the dried CSF.

Procedure of the diphenylamine reaction: 2 ml each of the samples was placed in glass-stoppered tubes. 3 ml of diphenylamine reagent was added: the contents were mixed by shaking. The tubes were capped with glass-stoppers and heated in a vigorously boiling water bath for 30 minutes exactly. The tubes were removed and cooled with running water. The reagent blank tubes were run with each procedure and contained of 2 ml of redistilled water or 5 per cent trichloroacetic acid solution and 3 ml of diphenylamine reagent. The reagent blanks were treated exactly like the samples at the same time. The reagent blank treated

was decanted into a small colorimetric tube and set to 100 per cent transmission at the wave length of $530m\mu$ on a Hitachi photo-electric spectrophotometer (TYPE EPU-2A) and the transmission of the samples was read against the reagent blank.

Reagents:

i) Diphenylamine reagent¹⁸⁾; 1 g of diphenylamine was placed in 100 ml graduated cylinder. 100 ml of pure glacial acetic acid was added. Then, 2.75 ml of concentrated sulfuric acid was added: the contents were mixed.

ii) Absolute ethanol.

iii) Saturated and 5 per cent trichloroacetic acid solution.

iv) Redistilled water.

RESULTS

1) Experiment 1 (with sample 1): The blue or indigo-blue coloration appeared and the extinction value was small. As partly shown in Table I, the purple coloration expected appeared rarely. An extinction curve of the cases colored purple is shown in Figure 1.

2) Experiment 2 (with sample 2): The blue or indigo-blue coloration appeared more intensely than in the experiment 1. The purple coloration appeared extremely rarely.

3) Experiment 3 (with sample 3): In this experiment, the reaction produced a clear purple coloration. When the samples were concentrated to this grade, the extinction value could be estimated with confidence.

TABLE I. Extinction Value in Various CSF Samples by the Diphenylamine Reaction

Case No.	Whole CSF	Supernatant (I)*	Supernatant (II)** (Concentration grade)	Whole CSF plus saturated trichloroacetic acid (ml)
1	.022	.140	∞ (10)	.100 (0.3)
2	.072	.116	∞ (10)	.105 (0.3)
3	.068	.096	∞ (10)***	.088 (0.1)
4	.090	.137***	∞ (10)***	.125 (0.1)
5	.045	.065	.312 (4)	.070 (0.1)
6	.046	.065	.323 (4)	.075 (0.1)
7	[.055]****	.129	[.308] (4)****	.089 (0.1)
8	.110	.160	.196 (2)	.132 (0.1)
9	.096	.125	.169 (2)	.120 (0.1)
10	.066	.110	.296 (4)	.092 (0.1)
11	.092	.096	.238 (4)	.095 (0.1)

* CSF samples freed of the protein by the trichloroacetic acid method.

** Protein-free CSF: The protein in the CSF dried *in vacuo* was removed with 5 per cent trichloroacetic acid solution.

*** The coloration with a tint of purple. (The main coloration was blue).

**** The main coloration was purple.

Fig. 1. Extinction curves by the diphenylamine reaction with the samples obtained from one subject at once.

————: Serum Niazi-State's fraction (as a control) (Extraction into 2 ml of 5 per cent trichloroacetic acid solution from protein in 0.25 ml serum)

—x—x—x—: Whole CSF. (Experiment 1)

.....: Dried CSF sample with added 5 per cent trichloroacetic acid solution. (Experiment 9)

ooooooo: Dried CSF sample dissolved in redistilled water. (Experiment 10)

4) Experiment 4 (with sample 4): The reaction produced a clear purple coloration as in the experiment with Niazi-State's fraction made from serum protein. In this experiment, the extinction value was readily estimated, when the sialic acid was extracted from the total protein in 10 ml of CSF into 1.5 or 2 ml of 5 per cent trichloroacetic acid solution.

5) Experiment 5 (with sample 5): The reaction produced mainly a blue or indigo-blue coloration, rarely with a tinge of purple, while a purple coloration with a tint of blue appeared extremely rarely. As shown in Table I, the extinction value was larger than in experiment 1.

6) Experiment 6 (with sample 6): As partly shown in Table I, a purple coloration appeared in a small part, while in the large part was produced a blue or indigo-blue coloration. An extinction curve of the cases is shown in Figure 2.

The tests in experiments 5 and 6 were repeated 90 times. The main coloration with purple appeared 6 times and the coloration with a tint of purple 17 times, while in 67 cases, dark blue appeared with a tinge of green or indigo-blue. There was no case showing clear purple coloration as seen in experiment 4.

Fig. 2. Extinction curves by the diphenylamine reaction.

.....: Dried CSF sample with added 5 per cent trichloroacetic acid solution*.
(Experiment 9)

×××××××: CSF freed of protein with 5 per cent trichloroacetic acid solution.* (Experiment 6)

— · — · — · —: Dried protein-free CSF (by the ethanol method) with added 5 per cent trichloroacetic acid solution.* (Concentration grade was twofold.) Experiment 8)

————: Dried protein-free CSF (by the ethanol method), dissolved in redistilled water. (Experiment 7)

△△△△△△△: Protein-free CSF by the trichloroacetic acid method. (Experiment 5)

* These samples were obtained from one subject at once.

7) Experiment 7 (with sample 7): Purple coloration with a tint of blue appeared in many cases. Compared with the concentrated samples freed of protein by the trichloroacetic acid method, the blue or indigo-blue color appeared very faint. As shown in the extinction curve in Figure 2, the extinction value was smaller than in the experiment with samples in the same concentration prepared by the trichloroacetic acid method.

8) Experiment 8 (with sample 8): The main coloration was purple. Against our expectation, there appeared no case with the main coloration of blue or indigo-blue. But the blue coloration was more intense and the extinction value was greater than in experiment 7. The extinction curves of some cases, as shown in Figure 2, resembled the curve obtained in experiment 6.

9) Experiment 9 (with sample 9): The main coloration was blue or indigo-

blue with a tint of purple in most cases. The extinction curves of the reactions are shown one by one in Figures 1 and 2.

10) Experiment 10 (with sample 10): The main coloration produced was purple with a tint of blue. In a small part of the cases, it was dark purple and/or blue-indigo color. An extinction curve of the cases is shown in Figure 1.

COMMENT

In reference to the colorimetric determination adopted for estimation of sialic acid, 7 methods have been published and the most employed one is Bial's orcinol reaction. It has been recognized,¹⁹⁻²⁷⁾ however, that diphenylamine reaction is the most suitable one, for determination of serum sialic acid. We^{10,28-30)} have also used it for estimating serum sialic acid. There are, however, no reports in which sialic acid in the CSF is estimated by this reaction. Therefore, we investigated the serviceability and the problems of this method. Besides, some reports³¹⁻³⁶⁾ state that the free or dialyzable sialic acid exists in a larger content than the protein-bound one in CSF. We tried to test this assertion, too.

It is characteristic of sialic acid that it produces a clear purple color by diphenylamine reaction. The whole CSF samples, however, produced mostly blue or indigo-blue coloration, which was not expected for sialic acid, and the purple coloration in these experiments appeared only in a small part and the extinction value was very small. The whole CSF sample with added trichloroacetic acid produced deeper blue coloration and the extinction value was higher in this case. The CSF sample freed of protein with trichloroacetic acid showed the same tendency, too. Namely, the main coloration of purple appeared extremely seldom and even the coloration with a tint of purple appeared only rarely. The extinction value of these samples was larger than that of whole CSF.

On the other hand, the samples of protein extracted from CSF produced the clear purple as expected.

Also the samples of whole CSF dried *in vacuo* mostly showed the purple coloration. It is produced not only in the solution with added redistilled water but also in the solution with added trichloroacetic acid. We cannot understand the reason. But in these experiments, most the cases produced a coloration with a tint of blue and the samples containing trichloroacetic acid produced this blue coloration more intensely than the samples free of trichloroacetic acid. With the samples with protein precipitated away by ethanol method, the reaction produced the same finding. As shown in Figures 1 and 2, the extinction value was far larger when the sample contained trichloroacetic acid than in the case when the sample did not contain it.

Looking over Figure 1, the extinction curves indicate a difference from the curve made with Niazi-State's fraction of serum protein. In the former curves, the peak at the wave length of 660m μ , certainly^{25,37)} implies the blue coloration.

It is also suspected that more intense coloration is produced by the presence of trichloroacetic acid in the system of reaction and the extinction value at $530\text{ m}\mu$, becomes larger by the same condition. With Niazi-State's fraction, the peak at $530\text{ m}\mu$, becomes conspicuous owing to the low peak at $660\text{ m}\mu$. It may be that sialic acid is much more hydrolyzed by heating for 15 minutes than the interfering substances, as indicated in many reports,³⁸⁻⁴¹⁾ for sialic acid is bound to the end of protein. The result of following experiment demonstrates it. Namely, in the case the extraction and the diphenylamine reaction were performed at the same time by Ayala's method,¹⁹⁾ the longer the heating time, the more intense the blue coloration and the larger the extinction value at $650\text{ m}\mu$.

Then, we must notice the upper three curves obtained with samples obtained from one subject at the same time, in Figure 2. There is a very small difference between the two curves made from the results of experiments 6 and 9. It looks as if we must support the opinion that the large part of the sialic acid in CSF exists in the free form. But we hold a different view. We think it is a matter of course that the dried CSF sample with added 5 per cent trichloroacetic acid solution should show the largest extinction value. But the difference is too large between the extinction value obtained from the sample freed of protein by addition of the trichloroacetic acid to the dried CSF (experiment 6) and that obtained from dried CSF freed of protein by ethanol method and dissolved in the 5 per cent trichloroacetic acid solution (experiment 8). If this difference is owing to the presence of mucoprotein, which is precipitated more by ethanol than by trichloroacetic acid, we cannot interpret¹²⁾ the difference between the two curves made from the samples of whole CSF and protein-free CSF.

As a whole, it is difficult to say that the large part of the sialic acid in CSF is in free form, though there is a possibility that some free sialic acid does exist in the CSF.

What we are sure at present is that there are no reliable methods for determination of the sialic acid content in CSF except the protein-bound portion. We think it is necessary to investigate the increasing blue coloration and the higher extinction value at $660\text{ m}\mu$ due to trichloroacetic acid, in diphenylamine reaction of CSF samples. That is to say, it is necessary to study the method to diminish the interfering non-characteristic reaction. We think some sacrifice of sensitivity may be unavoidable for this purpose.

By the way, though the extinction value tends to rise with after the diphenylamine reaction, the degree of rise is slight. Therefore, it is recognized that diphenylamine reaction leads to stabler results than the direct Ehrlich reaction¹³⁾ or Bial's reaction.¹²⁾

SUMMARY

We examined the diphenylamine reaction for determination of sialic acid in

various samples of the human cerebrospinal fluid. It is recognized that the reaction is an unsatisfactory method for this purpose, except in estimating the acid content in cerebrospinal fluid protein or Niazi-State's fraction. In the cases trichloroacetic acid is present in the samples or the reaction system, the blue or indigo-blue coloration tends to intensify, even when the main coloration is purple, and the extinction value is heightened in its presence, not only at 530m μ , but also at 660 m μ . It is necessary to investigate this tendency furthermore.

Though there is a possibility that some free sialic acid exists in the cerebrospinal fluid, it might be denied that free sialic acid exists in a quantity several times larger than protein-bound sialic acid.

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