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CHROMATOGRAPHIC PROCEDURES THAT ARE PROVING USEFUL IN RESEARCH ON RIBONUCLEASES

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CONTENTS

1. INTRODUCTION

The organizers of this symposium, and most of the members of this audience, have special memories of Arne Tiselius. I recall that on my first trip to Europe, in 1949, Uppsala was a key city on my itinerary. A young American on his initial journey overseas might be expected to think first of the major capitals in this part of the world; yet my thoughts turned especially toward the scholarly town of Uppsala and the University which it hosts. The presence of Arne Tiselius and the contributions that he was making to the methods of chromatography in those years was the source of my affinity for Uppsala. I came to consult him in reference to the experiments that William Stein and I were conducting at The Rockefeller Institute on the chromatography of amino acids, peptides, and proteins. A conversation with Arne Tiselius in Uppsala or in New York was always most stimulating to us, both personally and in terms of the roles of separation methods in biochemistry and the principles from which they could evolve. The spirit of this conference in Sweden stems from his leadership. The rate of progress in the development of separation techniques in the past thirty years has exceeded all expectations and a number of the innovations have come from the laboratories of the Institute of Biochemistry in Uppsala.

2. CHROMATOGRAPHIC PROCEDURES

Methods are a means to an end, not an end in themselves, and the main theme of my specific contribution this morning will be to give a few examples of the ways in which some of the current procedures for the separation of proteins are helping us to learn more about ribonucleases. In the interest of simplicity I will start with the elegantly simple technique of gel filtration which has rapidly grown into one of the most widely used methods in protein chemistry. It was during my visits to Uppsala in the 1950s that I first learned of the invention of gel filtration and Sephadexes by Porath and Flodin[1]. We are among the many who promptly found that the method

cculd be extremely useful. In the special journal volume dedicated to Arne Tiselius in 1962, Crestfield et al.[2] had the opportunity to describe (Fig. 1) the fractionation by gel filtration of the aggregates formed when bovine pancreatic ribonuclease is lyophilized from 50% acetic acid. The main product was a dissociable dimer of molecular weight about 28,000. Since then, renewed interest in dimers of ribonuclease has grown from the researches of Leone and D'Alessio and their colleagues[3,4] on a ribonuclease in bovine seminal plasma which is homologous in primary structure to the pancreatic enzyme of the same species but is a dimer cross-linked by two –S–S– bonds; Libonati and Floridi[5] found that the dimeric enzyme is more active toward double-stranded RNAs than is the pancreatic monomer. This observation prompted us to study the cross-linking of the pancreatic monomer by diimido esters with the aim of obtaining a stable dimeric derivative which would have increased activity toward poly A·poly U. Hartman and Wold[6] in 1967 and Wang et al.[7] in our laboratory used gel filtration of the same type illustrated in Fig. 1 to isolate the dimeric fraction from the reaction products. The most active dimeric derivative had about eight times the activity of the monomer toward the double-stranded substrate. But that is only a modest increase in activity. How might we gain a greater increase? D'Alessio et al.[4] and Libonati et al.[8,9] developed the thesis that the number of basic charges in a ribonuclease was the important variable in the action toward double-stranded substrates. Accordingly, Wang and Moore[10] undertook to use dimethyl suberimidate to cross-link ribonuclease to a polyamine, such as spermine, which has a special affinity for nucleic acid. In this instance, the gel filtration was used initially to remove any ribonuclease dimer from the desired spermine–ribonuclease fraction,
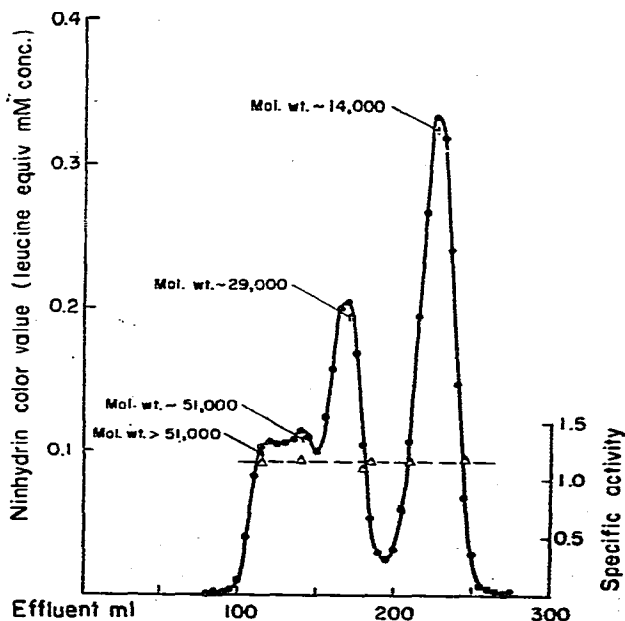

Fig. 1. Separation of ribonuclease A and its aggregates on a 2 × 143 cm column of Sephadex G-75 with 0.2 M sodium phosphate buffer (pH 6.47) as the eluent. The molecular weights were determined by ultracentrifugation. From ref. 2.
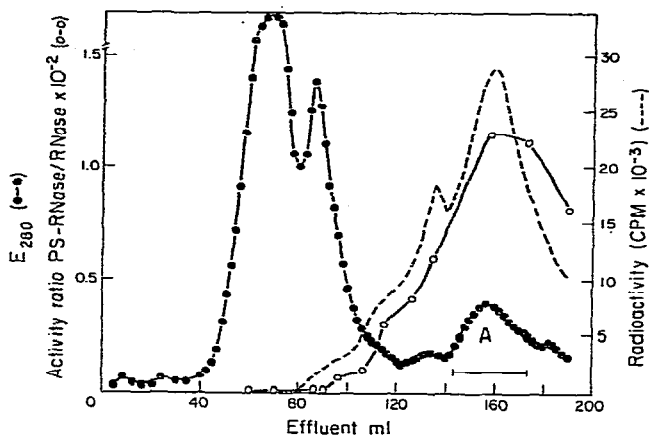
Fig. 2. Fractionation of polyspermine–RNases on carboxymethyl–Sephadex (C-50). Column, 0.9 × 60 cm. Elution was with a linear gradient of pH and NaCl concentration formed with 100 ml of 0.1 M phosphate buffer of pH 6.4 and 100 ml of 0.1 M phosphate buffer of pH 8.0, 0.7 M in NaCl. Activity was measured toward poly A·poly U. From ref. 7.

which was then submitted to ion-exchange chromatography on carboxymethyl–Sephadex (Fig. 2). The result was a product (from Zone A) which had an average of 8 spermine residues (measured by ^{14}C) per molecule of ribonuclease. The activity was about 100 times the activity of the native enzyme toward poly A·poly U and nearly 400 times the activity toward the hybrid substrate poly rU·poly dA. A natural double-stranded RNA from reovirus 3 could be completely converted to acid-soluble nucleotides in 10 min by polyspermine–ribonuclease.

In our early experiments on pancreatic ribonuclease, one of the approaches was to see whether we could lower the activity of the enzyme by specific derivatizations; in this way we plotted residues which were probably at or near the active site of the catalyst[11]. It is also informative to see whether you can make an enzyme more active than the native molecule. The cross-linking experiments with ribonuclease have been one step in this direction.

In the early days of our studies on the chromatography of proteins on ion exchangers, William Stein and I frequently discussed the theory of the process with Arne Tiselius. We were then seeking to obtain finite distribution coefficients which would permit a protein to be eluted by a single buffer. This objective was achieved in cooperation with Werner Hirs in 1951[12,13]; ribonuclease (Fig. 3) was eluted from the polymethacrylic acid resin Amberlite IRC-50 just as simply as was an amino acid from the same exchanger or from the sulfonated polystyrene resin Dowex 50 (ref. 15). But ribonuclease is a relatively small protein; Arne Tiselius discussed with us the mathematics of all-or-none adsorption that would be approached as multipoint attachment increased with larger proteins and the probable need for increases in pH and ionic strength for successful elution of large proteins from ion exchangers[16,17]. In 1954 he noted[16] that the rate of elution of polyvalent proteins will tend to change from an R_F value of zero to an R_F of one in a very narrow range of buffer concentration. In that article he also suggested that hydrophilic matrices might be preferable to hydrophobic ones (such as polymethacrylates) for many protein separations. The
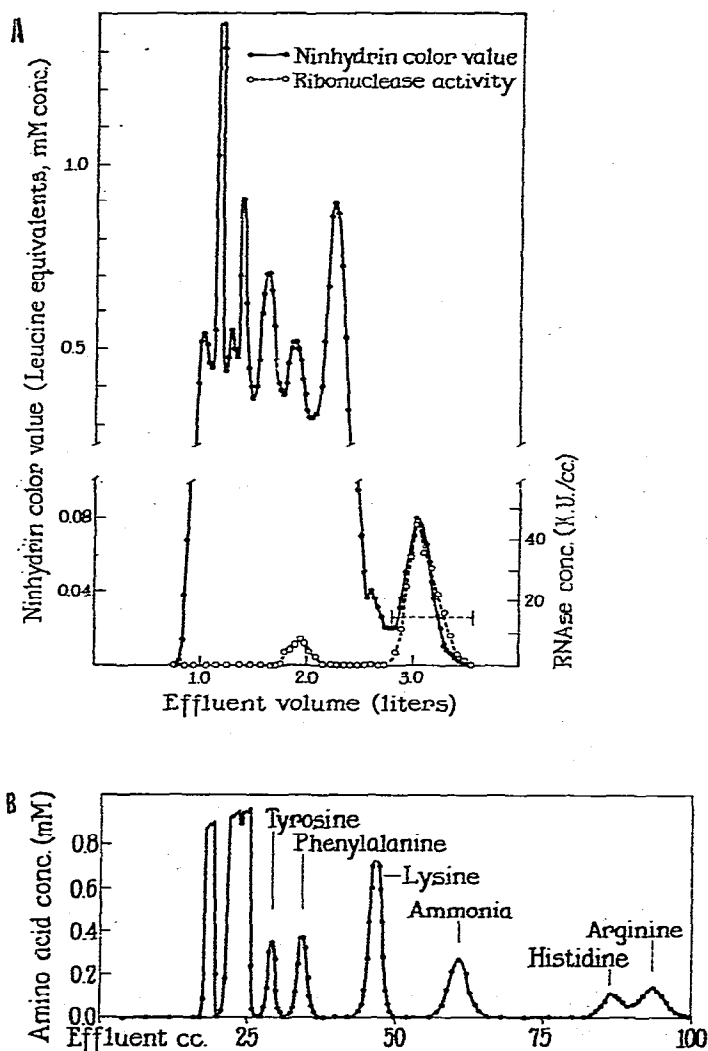
Fig. 3. (A) Chromatography of a 0.25 M sulfuric acid extract of comminuted bovine pancreas on the carboxylic acid resin IRC-50. Eluent, sodium phosphate buffer (0.2 M, pH 6.47); preparative column, 7.5 × 60 cm. (B) Chromatography of amino acids on the same resin (0.9 × 60 cm) with 0.5 M citrate buffer (pH 5.4). From ref. 13. The open circles give the ribonuclease activity; the smaller active peak (RNase B) was later characterized as a glycosylated derivative of RNase A by Plummer and Hirs[14].

exciting introduction by Peterson and Sober in 1956[18] of ion exchangers prepared from cellulose opened a whole new era in protein chromatography, in which exchangers based on the dextrans developed in Uppsala, such as the carboxymethyl-Sephadex used in Fig. 2, are among the most common reagents in protein chemistry.

Arne Tiselius[16], in the course of considering adsorbents for proteins, systematically explored hydrophilic gels of calcium phosphate in 1954 with the aim of making the adsorbent more generally useful. Ten years later, when we came to study

the homogeneity of pepsin[19] we noted that Tiselius *et al.*[20] in their report on the use of hydroxyapatite columns for a number of proteins, mentioned that in 1861 Brücke[21] reported that pepsin activity could be adsorbed and eluted from calcium phosphate. Brücke's research takes us back only about 100 years, which is not far relative to the 500 years of the University of Uppsala which we commemorate on this occasion. Chromatography of commercial pepsin on hydroxyapatite with increasing concentrations of phosphate gave us the chromatogram shown in Fig. 4; the extent of the heterogeneity prompted us to look for improved ways of preparing pepsin for structural study. A decade later, the same adsorbent was helpful in the experiments of Arabinda Guha in our laboratory on the purification of a phosphodiesterase from bovine brain (Fig. 5).
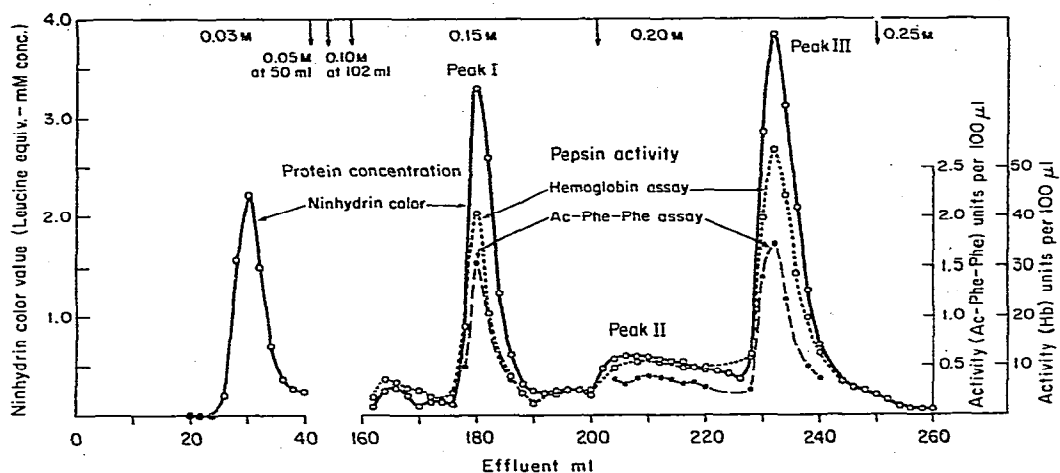

Fig. 4. Chromatography of commercial pepsin on a 0.9 × 40 cm column of hydroxyapatite. Phosphate buffers (pH 5.7) of increasing molarity were used as eluents. From ref. 19.
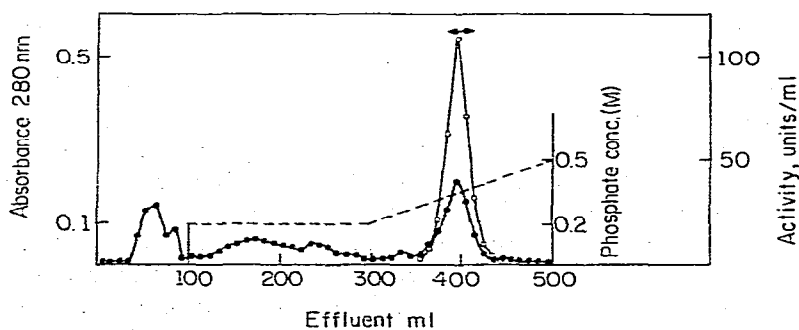

Fig. 5. Chromatography on hydroxyapatite of 2′,3′-cyclic nucleotidase from bovine brain; the sample was a partially purified fraction obtained by initial chromatography on carboxymethyl-Sephadex. The column (2 × 10 cm) was eluted with sodium phosphate buffer (pH 6.8) of increasing molarity. The open circles give the enzymic activity. From ref. 22.

The initial steps in the purification of 2',3'-cyclic nucleotidase from brain[22] are special because the enzyme is initially insoluble. In order to utilize the potential resolving power of chromatography for the purification of proteins, the protein has to be brought into solution. Much of the progress in enzyme chemistry in recent decades has been with soluble proteins available in gram quantities. Advances in the current decade are being made with many enzymes that are present in much smaller amounts and that frequently are membrane-bound. It is this realization that is stimulating the scaling-down of the methods of amino acid analysis to the nanomole range[23] and that has prompted us to see whether we could arrive at methods that might be helpful in the isolation of some initially insoluble enzymes.

Detergents are frequently employed for solubilization; Drummond et al.[24], in their initial studies on 2',3'-cyclic nucleotidase, used 6% Tween 20. But if there is a way to avoid detergents, we would prefer to be able to study the chemical and physical properties of an enzyme in plain aqueous solution. We have tried many combinations of conditions for the solubilization of the 2',3'-cyclic nucleotidase in brain; one approach which is working is summarized in Table 1.

TABLE 1

STEPS IN THE SOLUBILIZATION AND PARTIAL PURIFICATION OF 2',3'-CN 3'-ASE (FROM REF. 22)

The starting material was 20 g of an acetone powder prepared from about 100 g of bovine brain white matter.

Step	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
1. Initial extract (1 M in GuCl*) after centrifugation	12 000	2.5	100
2. Supernatant (0.2 M in GuCl*) after 5-fold dilution of (1)	9600	2.5	80
3. Precipitate obtained by addition of $(NH_4)_2SO_4$	9000	12.6	75
4. Carboxymethyl–Sephadex chromatography	3200	106	28
5. Hydroxyapatite chromatography	2500	508	21

* Guanidinium chloride.

The starting product in this instance is white matter, since this tissue is richest in the enzyme. Investigators who have studied the isolation of myelin find that the 2',3'-cyclic nucleotidase is most closely associated with the myelin fraction, which is unusual, because most enzymic activities in myelin are very low. An acetone powder from bovine white matter is homogenized with 1 M guanidinium chloride at pH 6; the solution is also 1 mM in dithiothreitol and EDTA. More than half, but not all of the enzyme is thereby solubilized. If, from this solubilized portion we dialyze out the guanidinium chloride in one operation, the enzyme reprecipitates, and we are back where we started. But, if we carry out a fractional precipitation, a sort of "ammonium sulfate fractionation" in reverse, by reducing the guanidinium chloride concentration stepwise, precipitates are obtained at 0.8 M, 0.6 M, etc. The enzyme stays in solution down to 0.2 M, and if the precipitate obtained at this guanidinium chloride concentration is spun off, the remaining solution can then be dialyzed completely free of guanidinium chloride without precipitation of the enzyme. The test for solubility is

retention of activity in the supernatant solution after centrifugation at 100,000 g for 4 h. We hope that this approach may be useful with some other membrane-bound proteins.

Our interpretation of this experiment is that the fractionation has separated the enzyme from a component in the extract with which the enzyme has a tendency to aggregate. The enzyme obtained at this stage can be concentrated by precipitation with $(NH_4)_2SO_4$ and redissolved for gel filtration and ion-exchange chromatography. After passage over carboxymethyl-Sephadex and hydroxyapatite, a 200-fold purification has been achieved.

The final example of methodology that we will discuss today is the application of affinity chromatography in our laboratory to the isolation of a ribonuclease inhibitor from human placenta by Blackburn and Wilson[25]. The presence in mammalian tissues of an inhibitor of neutral ribonuclease of the pancreatic type was first studied by Roth[26]. The biological connotations of the inhibitor's presence have recently grown in interest as it has become apparent that the ability of such an inhibitor to repress ribonuclease action may have a role in preserving the integrity of mRNA and polyribosomes[27] and hence facilitate protein synthesis and cell proliferation both *in vitro* and *in vivo*. The technique of affinity chromatography is ideally suited to the search in a homogenate for an inhibitor of an enzyme that can be attached to a suitable support. In this instance, pancreatic ribonuclease has been coupled to Sepharose 4B by the cyanogen bromide method pioneered by Porath and Axén[28]. The coupling with ribonuclease has been most complete when the cyanogen bromide (100 mg/ml settled bed) was dissolved (*cf.* ref. 29) in a minimum volume of a water-miscible organic solvent [we used N,N-dimethylformamide, approx. 1:1 (w/v)] and the solution was added dropwise to the stirred Sepharose suspension. When 50 mg of RNase A were coupled to 40 ml of CNBr-activated Sepharose, the final product contained about 1 mg of RNase per ml of settled Sepharose and did not shed detectable enzyme activity in the eluents, provided Tris-buffer was not used. The deleterious effect of Tris and other amino-containing buffers on the stability of ligands attached to CNBr-activated Sepharose has been reported by Wilchek *et al.*[30] and by Tesser *et al.*[31].

Human placentas obtained within 30 min of normal term delivery were homogenized in buffered 0.25 M sucrose, 5 mM in dithiothreitol and 1 mM in EDTA. A 35–50% ammonium sulfate precipitate was prepared, the ammonium sulfate was removed by dialysis, and a preliminary fractionation was conducted on DEAE-cellulose. The inhibitor is an acidic protein which is retarded on the exchanger at pH 7.5. The fraction containing the inhibitor was equilibrated with 45 mM phosphate buffer at pH 6.4 and applied to a column of RNase–Sepharose. The inhibitor was strongly bound; elution was accomplished (Fig. 6) by decreasing the affinity of the inhibitor for the enzyme by lowering the pH to 5.0, increasing the salt concentration to 3.0 M in NaCl, and including glycerol (15%, v/v) to increase the stability of the protein. The inhibitor thus eluted gave a single band[25] upon SDS-gel electrophoresis. The overall recovery was 45% (Table 2). The retention of activity was dependent upon the use of both dithiothreitol and glycerol.

The inhibitor is isoionic at pH 4.6, has a molecular weight of about 51,000, and forms a 1:1 molecular complex with RNase A with a K_i of $3 \cdot 10^{-10}$ M. Affinity chromatography has thus made available the pure inhibitor in a form that can be used
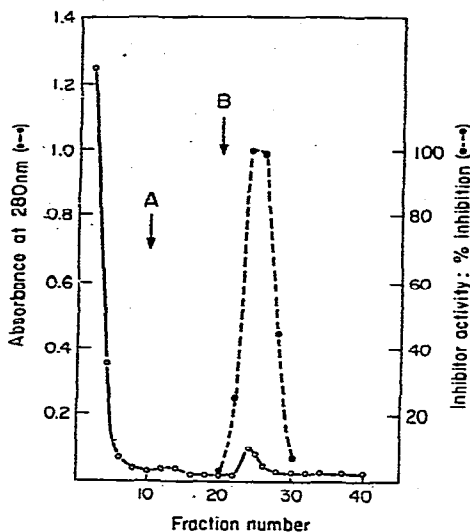
Fig. 6. Affinity chromatography of the ribonuclease inhibitor from human placenta on RNase–Sepharose (settled bed 8 ml). The sample was a partially purified fraction from a DEAE-cellulose column. The initial eluent was 45 mM phosphate buffer (pH 6.4). At A the buffer was made 0.5 M in NaCl. At B elution was initiated with 20 mM acetate buffer, pH 5.0, 3 M in NaCl and 15% (v/v) in glycerol. From ref. 25.

TABLE 2

PURIFICATION OF RNASE INHIBITOR FROM HUMAN TERM PLACENTA (FROM REF. 25)

Step	Total protein (mg)	Total inhibitor (units)	Specific activity (units/mg)	Recovery of activity (%)
1. Initial extract	16,000	400,000	25	100
2. 35–50% ammonium sulfate precipitate	2,327	320,000	137.5	80
3. 48,000 g, 1 h	1,646	320,000	194	80
4. DEAE-cellulose chromatography	308	240,000	780	60
5. Sepharose–RNase A affinity chromatography	1.8	180,000	100,000	45

in studies of its interaction with cellular RNase and its effects on RNase activity in *in vitro* translation and transcription studies.

These comments have illustrated some of the ways in which chromatographic methods, a number of which originated in Uppsala, have contributed to the isolation of proteins in our laboratory. The program of the First Tiselius Symposium includes reports on many experimental techniques that will have a direct bearing on the progress of biochemistry. The epoch in biochemistry covering the past thirty years has been one of great growth in our science. Methodology has had a key role in this process. The invention of new methods and new instruments and the cooperation of academic and industrial scientists in making supplies and equipment available to the

researcher have contributed to the practice and the art of separating molecules. The literature today emphasizes the accelerated rate of discovery of proteins and of elucidation of their structures. It is important to realize how much remains to be done. Protein chemistry today is in a stage of development that bears some similarity to the state of organic chemistry at the turn of the century. At that time there was great activity in expanding the volumes of Beilstein with characterizations of the myriad small organic compounds conceivable by man and nature. Today, in the polypeptide field, the atlas of protein structures is one volume; in the year 2000 it will be an encyclopedia of many volumes. And there will be a companion series on nucleic acids. Knowledge of macromolecular architecture is a prerequisite for fundamental understanding of the intricate synergisms of living systems and separation methods which we will discuss this week will have an increasing role in the accumulation of such information.

3. SUMMARY

Examples are given of the uses of gel filtration, chromatography on calcium phosphate, ion-exchange chromatography, and affinity chromatography, in the course of researches on pancreatic ribonuclease, dimeric derivatives of the enzyme, the $2',3'$-cyclic nucleotide 3'-phosphohydrolase of brain, and the inhibitor of ribonuclease of the pancreatic type in the human placenta.

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