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Intercellular Adhesion

II. The Purification and Properties of a Horse Serum Protein that Promotes Neural Retina Cell Aggregation*

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Summary. When 8-day-old embryonic chicken neural retina is dissociated, the resulting single cells adhere to each other at a rapid rate at 37 °C, and slowly at 5 °C. However, the addition of horse serum substantially accelerates the rate of aggregation at 5 °C, although it shows no effect at 37 °C; the rate at 37 °C exceeded the maximum rate at 5 °C (i.e., in the presence of excess horse serum). The kinetics of the horse serum effect were investigated, and, based on these results, an assay was devised for the active component in the serum. The active protein, termed "neural retina aggregating protein" (NRP), was purified 48-fold from the serum, and at this stage appears close to homogeneity. The rate of aggregation of neural retina cells is significantly stimulated by adding 1 μg of purified NRP per ml. Purified NRP shows certain properties in common with horse macroglobulins (horse Immunoglobulin M, IgM), but there are also some important differences. For example, it cross-reacts with antibodies to horse IgM, it can be reduced to subunits with thiols, and it is of high molecular weight (about 1.6×10^6). However, NRP has a higher sedimentation value than IgM (22.4 S compared to 19 S), and purified IgM does not increase the rate of aggregation of neural retina cells at 100-fold the concentration of NRP required for optimum activity. Inhibitory activity was not detected at any stage of the purification of NRP from horse serum. However, other sera (chick and calf) were shown to reduce the normal slow rate of aggregation at 5 °C and to inhibit the effect of NRP. Some preliminary studies indicate NRP may be specific for neural retina cells. For example, the rate of aggregation of 5-day-old embryonic limb bud cells is unaffected by concentrations of NRP that are optimal for neural retina cells.

The accompanying paper [9] describes a quantitative procedure for determining the initial kinetics of cell aggregation, i.e., the rate at which single cells in suspension adhere to each other. The method measures the rate of loss of single cells which directly reflects the rate of formation of aggregates. An examination of the kinetics of this event shows that the following parameters influence the rate: initial cell density, speed of rotation of the flasks containing the single cell suspensions, divalent cations, and temperature. The rate of aggregation of the cells used for these studies, 8-day-old chicken embryo neural retina, was independent

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of inhibitors of protein synthesis and a variety of metabolic inhibitors; no effect of nucleotides could be demonstrated.

Using this quantitative kinetic approach, we noted that the retina cells aggregated rapidly at 37 °C, but very slowly at 5 °C. This is in agreement with the earlier reports [1, 8, 10] with the exception of that of Curtis and Greaves [3]. In addition, horse serum, a subject of much previous controversy (for review, *see* [2]), did not affect the rate of aggregation at 37 °C. However, a marked stimulation of adhesion was noted at 5 °C, and this effect led to the experiments reported below.

Analysis of the stimulatory effect of horse serum on the aggregation of embryonic neural retina cells at 5 °C indicated that the serum component responsible for the effect was a protein. Application of the quantitative assay permitted isolation of the protein in a highly purified form. As will be shown, the protein exhibits many of the properties of macroglobulins, although certain important differences can be detected. In addition, purified "neural retina aggregating protein" (NRP) does not appear to increase the rate of aggregation of limb bud cells at 5 °C.

Materials and Methods

Preparation of Cells. Single cell suspensions of 8- and 10-day-old neural retina cells were prepared as described previously [9]. Five-day-old limb bud cells were dissected from embryos obtained from white Leghorn eggs (Truslow Farms, Chestertown, Md.), and single cell suspensions were prepared in exactly the same way as described for the neural retina cells, except that filtration through Nytex was omitted.

Size Spectrum of the Cells. Ten-day-old neural retina cells were prepared, their size spectrum was determined with the Coulter Counter, and the data were recorded on the automatic plotter. It was found that the spectrum did not differ significantly from that described for 8-day-old neural retina cells [9]; the same instrument settings were therefore used.

The size spectrum of limb bud cells indicated that these cells had, on the average, twice the volume of the neural retina cell. After several separate experiments, we found that the major fraction of the single cell population could be determined at 1/amplification = 2, and 1/amperage = 0.707, with the upper and lower thresholds set at 20 and 60, respectively. This population represented 75% of the single cells in the total population. In order to determine whether 5-day-old limb bud cells aggregated similarly or differently from 8-day-old neural retina cells, the rates of aggregation of the two cell types were compared as described [9]; the same initial cell densities (0.8×10^5 cells/ml) were employed. The resulting slopes for the two cell types were not significantly different ($t = 0.023$).

Sera[1]. Two different batches of horse serum have been used: (1) Lot no. 7021325 from BBL, Cockeysville, Md., and (2) Control no. 18084A from Gibco, Grand Island, N.Y. The chicken serum (Lot no. 7021337) and calf serum (Lot no. 7071202) were both from BBL. All sera were stored at −70 °C and, before use, were heated at 56 °C for 30 min.

Media. All media and chemicals used in the assay system were from the same sources, and were prepared as described in the previous communication [9].

1 The concentration of serum employed in each experiment is designated mg/ml, referring to mg serum protein used per ml.

Other Chemicals. Diethylaminoethyl (DEAE)-cellulose (DE 23) was obtained from Whatman Co. Agarose 1.5 and 5 m was obtained from BioRad Laboratories (Richmond, Calif.).

Assay Methods. The use, operation, and settings of the Coulter counter, and the methods for determining rates of aggregation have been described [9]. Protein determinations were performed either by the method of Lowry, Rosebrough, Farr, and Randall [5], or by absorbance at 280 mμ, taking the extinction coefficient ($OD^{1\%}_{280\,m\mu}$) of 15.0 found for horse serum Immunoglobulin M (IgM [6]).

Results

The Effect of Horse Serum on Aggregation. It has already been demonstrated that horse serum does not affect the rate of aggregation of 8-day-old neural retina cells incubated at 37 °C [9]. At 5 °C, however, the slow rate of aggregation normally observed [9] was greatly accelerated in the presence of horse serum (Fig. 1). The initial rates of aggregation at both 5 and 37 °C were constant with time, and no lag was ever demonstrated. The slopes of the lines at 5 °C were related to the concentration of added horse serum; i.e., the rate was a function of horse serum concentration (Fig. 2). The slopes have been calculated as previously described [9]; over the range of 0.075 to 0.30 mg of horse serum per ml, the slopes are proportional to the concentration of horse serum. At the highest concen-

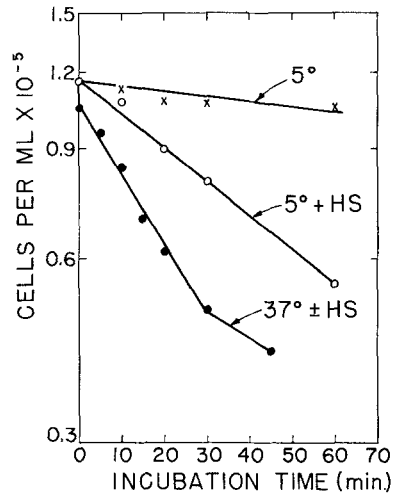

Fig. 1. The effect of horse serum on the rate of aggregation of 8-day-old chick neural retina cells. Two separate cell suspensions were prepared as described [9]. Cells from one suspension were incubated under standard conditions [9] at 37 °C at an initial cell density of 1×10^5 cells/ml in the presence or absence of 1 mg/ml horse serum (•–•–•). Duplicate flasks were removed at the indicated times, and the single cell concentration was determined as described [9]. The second suspension was prepared in the same way and incubated at 5 °C in the presence (o–o–o) and absence (×–×–×) of 1 mg/ml of horse serum. The initial cell density was 1.17×10^5 cells/ml. Duplicate flasks were removed at the indicated time points, and the single cell concentration was determined as described [9]

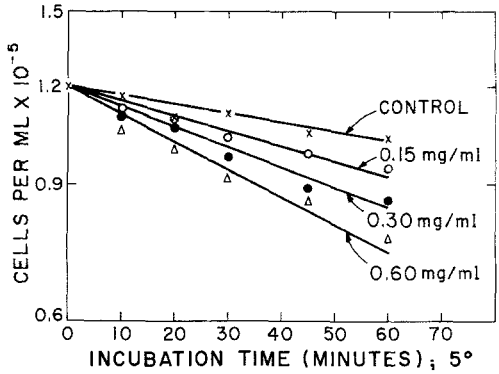

Fig. 2. The effect of horse serum concentration on the rate of aggregation of 8-day-old chick neural retina cells. Single cells were prepared as described [9] at an initial density of 1.2×10^5 cells per ml. The cells were incubated at 5 °C in the presence of one of the following concentrations of horse serum; none (×–×–×); 0.15 mg/ml (o–o–o); 0.30 mg/ml (•–•–•); and 0.60 mg/ml (Δ–Δ–Δ). Two flasks were removed at the each time point, and the number of single cells was determined as described [9]. The slopes and standard errors of the four lines (in the order described above) were: 0.0011, 0.0002; 0.0017, 0.0001; 0.0024, 0.0002; and 0.0030, 0.002, respectively

tration (0.60 mg/ml), the proportionality was lost; the cells were apparently saturated with horse serum, and no further increase in the rate was observed.

The kinetics of aggregation at 37 and 5 °C (in the presence of horse serum) exhibited two major differences: (a) The constant rate of aggregation, when determined as shown in Figs. 1 and 2, is extended for at least 60 min at 5 °C, whereas the rate was generally constant for only 30 min at 37 °C. (b) The maximum rate obtained at 5 °C (in the presence of horse serum) was much less than that observed at 37 °C when the same initial cell densities were used.

The fact that the rate of aggregation at 5 °C remained constant for 60 min permitted substantial simplification of the assay system. Rather than determining the slope based on data for a number of time points, a single time point (30 min) was routinely employed. The net difference between the control and the test system (referred to as the Δ in single cells) at 30 min was a measure of the effect of adding horse serum. The effect of increasing amounts of horse serum on the rate of aggregation, when expressed in this way, is shown in Fig. 3 a and b. The rate was related to the concentration of horse serum only over a narrow range (Fig. 3 b)[2], and substantiates the observation shown in Fig. 2. On the basis of these findings, it is possible to define a unit of aggregation activity, and thereby permit comparison of subsequent purification procedures. A unit was

2 The range can be extended when the results are treated in the standard manner (i.e., Fig. 2).

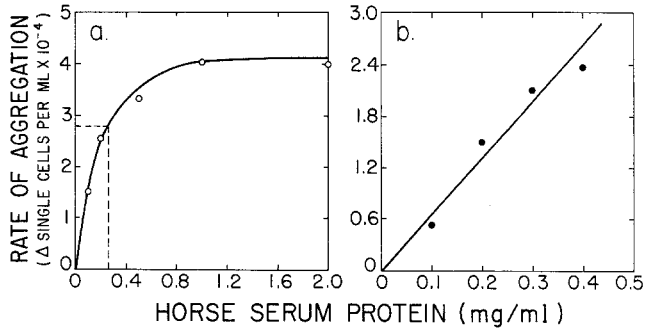

Fig. 3. (a) The effect of increasing horse serum concentration on the rate of aggregation of 8-day-old chick neural retina at 5 °C. The cells were incubated in the presence of the indicated concentrations of horse serum at an initial density of 1.3×10^5 cells per ml. Duplicate flasks were removed after 30 min, and the Δ in single cells was measured as described in the text. (b) The rate of aggregation at low horse serum concentrations. The initial part of the curve in Fig. 3a has been expanded to demonstrate the range of linearity. The other experimental details are the same as for Fig. 3a

defined as the amount of protein that resulted in the aggregation of 1×10^4 cells/ml (measured as the decrease in single cells) in 30 min, at 5 °C and 70 rpm under standard conditions [9]. Obviously, the specific activity of a protein fraction (units/mg protein) can only be determined if the assays are conducted where the rate of aggregation is proportional to protein concentration.

The following criteria were used to demonstrate that the observed loss in single cells at 5 °C was due to the aggregation (adhesion) of the cells, rather than to cell death or any other phenomenon.

1. Aggregates were clearly visible in flasks after 30 min of incubation at 5 °C.

2. No increase in the debris population occurred [9], nor was there a significant increase in nigrosin-positive cells at the end of the incubation period. This ensures that cell death had not occurred, and that there were no significant increases in the number of leaky or damaged cells.

3. The total volume of cells present initially can be calculated if the cells are considered as spheres with a mean diameter of 6 μ [9]. The total volume of cells recovered after incubation in horse serum at 5 °C equalled the total volume of cells in the original suspension and the volume of untreated (or control) cells (Table 1). The results in Table 1 also demonstrate that the loss of single cells was accompanied by increases in populations of aggregated cells, and that the number of aggregates was enhanced in the presence of horse serum.

Table 1. *Effect of horse serum on formation of aggregates by neural retina cells*[a]

Treatment	Volume (μ^3)						Total volume (μ^3)
	1/amplitude						
	1	2	4	8	16	32	
HH (control)	1,821	1,892	1,040	504	656	609	6,523
HH + 50 µl of horse serum	1,267	1,460	920	736	1,075	1,120	6,577

[a] The Coulter Counter was used to determine whether the disappearance of single cells from a suspension of 8-day-old chicken embryonic neural retina cells, prepared as previously described [9], corresponded to the formation of aggregates. The experiment was conducted under standard conditions [9], except that the temperature was maintained at 5 °C, and the experimental flask contained 50 µliters of horse serum. After 30 min, the number of single cells per ml was determined on an aliquot as described [9], with the instrument settings as follows: 1/amperage = 0.707; lower window, 20; upper window, 48; 1/amplification = 1. Particles larger than single cells (aggregates) were then counted by changing the 1/amplification control in a stepwise manner from setting 1 to 32 (the number of larger aggregates, beyond setting 32, was too small for accurate assay); at each setting, the particle number corresponds to the number of aggregates within a narrow range of volumes. Particle number was converted to total volume of particles at each setting by making the following assumptions: (1) The cells are spherical, with a mean diameter of 6 µ. (2) The cell volume does not change when it is incorporated into an aggregate.

Purification of the Active Component from Horse Serum. The purification procedure finally adopted was as follows. Horse serum (100 ml) was exhaustively dialyzed against distilled water (in this and all subsequent manipulations, the temperature was maintained between 0 and 4 °C). The serum was dialyzed for three consecutive 12-hr periods against 10-liter portions of distilled water. After each dialysis, the euglobulin precipitate (which contained the activity) was removed by centrifugation at 17,000 × *g* for 15 min. Each precipitate was triturated with 4 ml of 0.3 M NaCl, and allowed to stand overnight at 4 °C. The suspensions were combined and centrifuged at 17,000 × *g* for 15 min to remove undissolved material.

The supernatant from the previous step (14 ml) was applied to a column (95 × 4 cm) of Agarose 1.5 m which had been previously equilibrated in 0.3 M NaCl. The column was eluted with 0.3 M NaCl, and the active material, which was excluded from the gel, appeared as a sharp peak at the front (determined with formalinized *Escherichia coli*). The OD_{280} profile of the Agarose 1.5 m column is shown in Fig. 4; the active material (45 ml) was pooled and rapidly brought to 0.1 M NaCl by the addition of distilled water. This step resulted in the formation of a small precipitate but little, if any, loss in activity. It is important to note that overnight dialysis against 0.1 M NaCl to reduce the salt concentration caused substantial loss in activity.

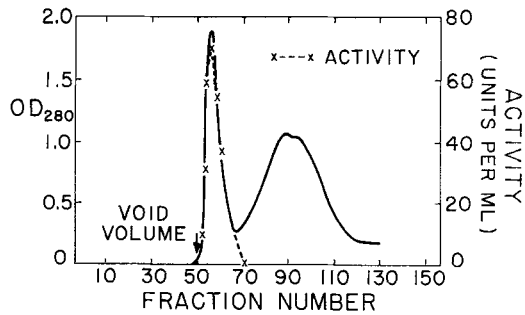

Fig. 4. The protein profile (OD_{280}) of the horse serum euglobulin fraction eluted from Agarose 1.5 m with 0.3 M NaCl. The neural retina aggregating activity is indicated (×–×–×); for other details see text

The diluted active fraction from the Agarose column was adsorbed to a column (50 × 2.5 cm) of DEAE-cellulose previously equilibrated in 0.1 M NaCl at pH 6.5, and the column was eluted with a linear gradient ranging from 0.1 to 0.35 M NaCl at pH 6.5. The activity was eluted as a large fairly symmetrical peak at approximately 0.2 M NaCl (Fig. 5). The contents of the tubes showing activity were pooled, and the salt concentration of the pooled material was accurately determined with a chloride electrode (Model RC-16B2 conductivity bridge, Beckman Instruments, Inc.). On the basis of this determination, the salt concentration was reduced to 0.1 M NaCl, and adsorbed on a small column of DEAE-cellulose. The activity was then concentrated by eluting in a small volume of 0.3 M NaCl.

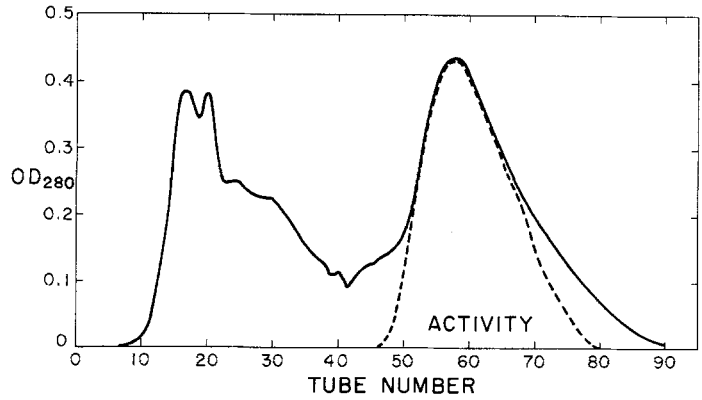

Fig. 5. The protein profile (OD_{280}) of the active pooled Agarose 1.5 m fraction eluted from DEAE-cellulose with a linear gradient of 0.1 to 0.35 M NaCl at pH 6.5. The activity is indicated (------); for other details see text

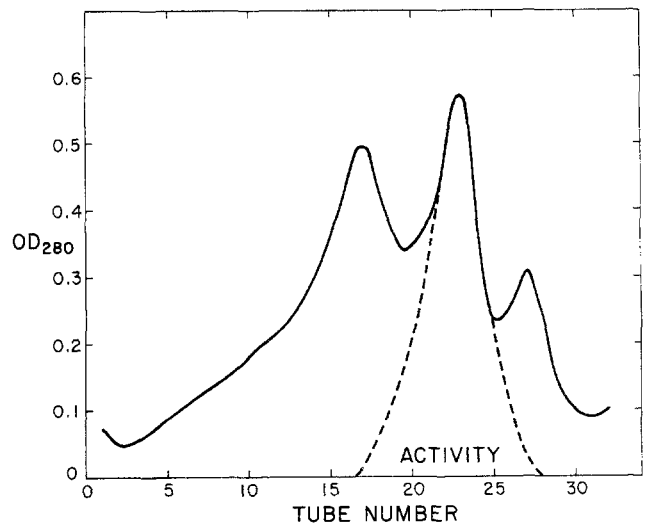

Fig. 6. The protein profile (OD_{280}) of the concentrated DEAE-cellulose fraction centrifuged in a gradient from 10 to 40% sucrose (w/v) in 0.3 M NaCl. Centrifugation was at 22,500 rpm in the SW-25 rotor for 19 hr. The fractions were collected automatically and the activity (------) was determined as described in the text

The concentrated DEAE-cellulose fraction was applied to a linear sucrose gradient (10 to 40% sucrose, w/v, in 0.3 M NaCl) and centrifuged at 22,500 × g (SW 25 head) for 15 hr in a model L Spinco ultracentrifuge. Fractions (0.8 ml) were collected, and the OD_{280} and activity of each fraction were measured. The OD_{280} profile showed three main peaks; the active material was located in the central peak (Fig. 6). The active fractions were pooled and concentrated with DEAE-cellulose as described above.

The results of a typical purification experiment are shown in Table 2. The major loss in activity occurred at the first step, and is thought to result from denaturation, since an appreciable quantity of the euglobulin

Table 2. *Purification of NRP*

Fraction	Specific activity (units/mg protein)	Purification factor	Recovery (%)
Horse serum	16.2	1.0	100
Dialyzed ppt.	44.0	2.7	47
Agarose 1.5 m	125	8	30
DEAE-cellulose	250	16	28
Sucrose gradient	750	48	15

precipitate was insoluble in 0.3 M NaCl. The active material was obtained in about 15% yield from the serum, and was about 48-fold purified.

In view of the conflicting reports concerning the presence of an inhibitor of cell aggregation in horse serum, it is important to emphasize that no inhibition was ever demonstrated in any of the horse serum fractions. Furthermore, the purified protein (NRP) exhibited full activity when added to horse serum in the standard assay, indicating again that the horse serum did not contain an inhibitor.

Properties of the Purified Protein

The purified material (NRP) obtained from the sucrose gradient was active at concentrations as low as 1 μg/ml. Fig. 7 shows the rate of aggregation of 8-day-old neural retina cells in the presence of 2.5 μg/ml of the purified fraction. The rate was constant for at least 90 min, and, as with crude horse serum, no lag was detected. The maximum rate of loss of single cells to form aggregates (3 to 4×10^4 cells/ml per 30 min) was the same with both the crude and purified fractions (*compare* Figs. 3a and 8).

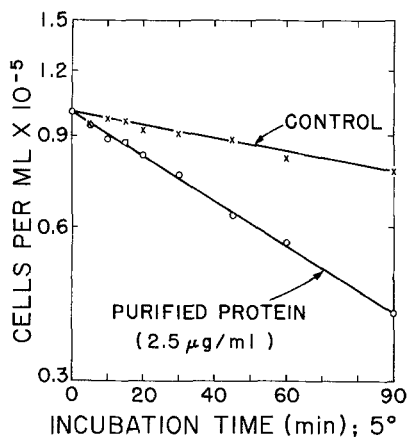

Fig. 7

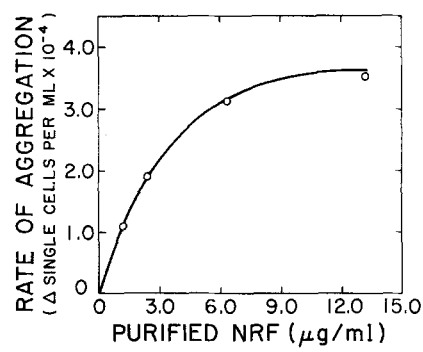

Fig. 8

Fig. 7. The effect of 2.5 μg/ml of purified NRP (sucrose gradient fraction) on the rate of aggregation of 8-day-old chick neural retina cells. Duplicate flasks were removed at the indicated time points from both control [no added NRP (×–×–×)] and NRP-containing flasks (o–o–o), and the number of single cells was measured as described [9]

Fig. 8. The effect of increasing the concentration of the purified NRP (sucrose gradient fraction) on the rate of aggregation of 8-day-old neural retina cells at 5 °C. The cells were incubated in the presence of the indicated concentrations of NRP at an initial density of 1.3×16^5 cells/ml. Duplicate flasks for each protein concentration were removed after 30 min, and the Δ was measured as described in the text

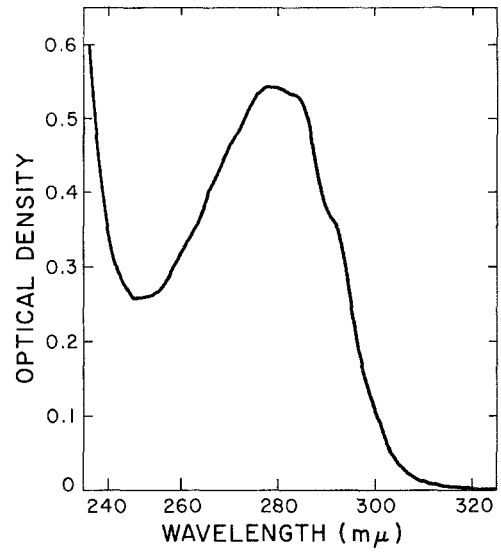

Fig. 9. Ultraviolet absorbtion spectrum of NRP (sucrose gradient fraction) recorded by a Carey model 15 recording spectrophotometer

The ultraviolet light absorption spectrum of a sample of purified NRP (sucrose gradient fraction) is shown in Fig. 9. The spectrum was typical of a protein containing the normal complement of aromatic amino acid residues; A_{280}:A_{260} was 1.7.

In 0.2 M NaCl, the activity was stable to storage for months at −20 °C, and to freezing and thawing. The activity was lost when purified NRP was dialyzed against water, and was also destroyed (85%) by heating for 3 min at 100 °C. The protein also lost activity when filtered through a 0.22-μ Millipore filter. Since the active species appears in the void volume of Agarose 1.5 m, it must be very large relative to the size of the usual serum proteins. This result suggests a molecular weight of $\geqq 1.5$ million. NRP did not migrate in $7\frac{1}{2}$% acrylamide gel, and it was stationary on cellulose acetate electrophoresis at pH 8.6 in Tris buffer. The active protein was retarded when chromatographed on Agarose 5 m. On the basis of the elution profile from Agarose 5 m (the void volume was established with formalinized *E. coli*) and the dimensions of the column, the molecular weight was estimated to be approximately 1.6×10^6. The large size of the molecule was further substantiated by ultracentrifugal analysis; an $S_{20\,w} = 22.4$ was obtained (using $\bar{v} = 0.723$ for horse macroglobulin [7]). The results of one ultracentrifugation experiment, using the concentrated DEAE-cellulose fraction in 0.3 M NaCl, is shown in Fig. 10. Although a smaller (10.2 S) and heavier (33.8 S) contaminating species was obviously

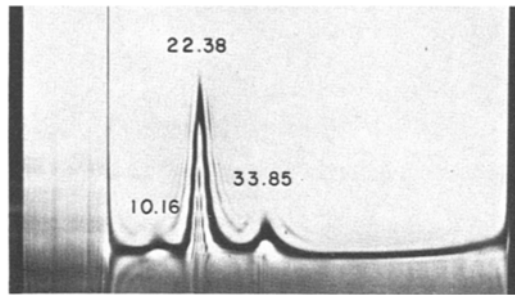

Fig. 10. Ultracentrifuge pattern of a sample of the concentrated DEAE-cellulose fraction (2.2 mg/ml) contained in 0.3 M NaCl. The frame (bar angle 50 °C) was taken 20 min after the start of the run which was at 5 °C in the Beckman model E ultracentrifuge. The material is sedimenting from left to right, and the S20w values of the peaks from left to right were 10–16S, 22–38S, and 33–85S, respectively

present, the active species was concluded to be the material sedimenting at 22.4 S, since less pure fractions contained much more of the heavier species, but exhibited lower specific activities. Active fractions have also been obtained that contained only the 22.38 S species, and these were used for the experiments described in Figs. 7, 8, and 9, and for the immunoelectrophoretic studies described below.

The large size of the active species and its fractionation characteristics suggested that the active material was probably a macroglobulin (IgM)[3]. This conclusion was supported by the following experiments: (a) In a single experiment, the subunit structure of the molecule was examined by using the technique of reduction and alkylation commonly employed with macroglobulins, and it indicated that the molecule contained disulfide bonds; the single symmetrical peak obtained with the native molecule on Agarose 1.5 m chromatography was lost, and a broader peak of lower molecular weight was obtained. When the lower molecular weight species were concentrated (DEAE-cellulose) and then examined by acrylamide ($7\frac{1}{2}$%) disc gel electrophoresis, several bands were detected. Furthermore, the smaller molecular weight material retained cell aggregating activity. (b) A close similarity between the purified NRP and macroglobulins was clearly demonstrated by immunoelectrophoretic techniques. As shown in Fig. 11, two separate aliquots of horse serum and one of purified NRP (which showed only the major peak in the ultracentrifuge) were subjected to electrophoresis in 1% agarose (Seakem, Marine Colloids, Inc.) containing 0.1 M barbital buffer, pH 8.6, for 1 hr. The adjoining troughs were

3 Nomenclature for human immunoglobulins. *Bull. World Hlth. Org.* 1964. **30**:447.

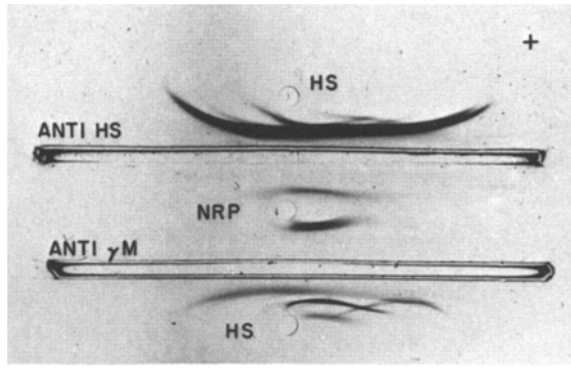

Fig. 11. The immunoelectrophoresis of horse serum (HS) and purified NRP (sucrose gradient fraction). Two 5-liter aliquots of HS and one 5-liter aliquot of NRP were placed in the wells cut in 1% Agarose in 0.1 M barbital buffer, pH 8.6. Electrophoresis, in 0.1 M barbital buffer at pH 8.6, was run at 15 ma for 1 hr. Either anti-horse globulin antibodies (anti-HS) or goat anti-horse IgM antibodies (anti-γM) were placed in troughs and allowed to diffuse for 24 hr at 25 °C

then filled with either rabbit anti-horse globulins (Pentex Co.) or with goat anti-horse macroglobulin (IgM); the latter was kindly supplied by Dr. John Cebra. After diffusion overnight at room temperature, the purified NRP showed a single precipitin band with both the rabbit anti-horse serum globulins and the goat anti-horse IgM globulin. Horse serum showed several precipitin bands with the goat anti-horse IgM antibodies, but one of the bands corresponded in shape and distance of migration to the purified NRP. The immunoelectrophoretic results, therefore, strongly suggest that NRP is either a macroglobulin or a closely related species. The results also indicate that the purified NRP is immunoelectrophoretically a single component, and we may conclude that the purified preparation is at a stage of purity approaching homogeneity.

Specificity of NRP

Conceivably, the effect of NRP on the embryonic chicken neural retina cells could result from nonspecific interactions between the large protein molecule and the cells, thereby promoting aggregation. This possibility appeared untenable in view of the fact that horse serum presumably contains a variety of proteins that could react similarly, i.e., in this nonspecific manner. In no case, at any step in the purification, was there any suggestion of more than a single active factor. The hypothesis of "nonspecificity" was examined more closely by testing the cells with similar proteins, and NRP has been tested with other cell types to determine if the activity with neural retina cells is specific.

The Effect of Other Antibodies on Neural Retina Cells. Two types of antibodies were tested with the embryonic neural retina cells: (a) In view of the close similarity of NRP to IgM, a purified preparation of the latter was tested in the standard assay system. The IgM was a 19 S macroglobulin obtained from horse serum, directed against a purified polysaccharide antigen from *Diplococcus pneumoniae.* The purified IgM was tested at concentrations up to 100 times that required for maximum activity by NRP, and the IgM showed no detectable activity. (b) A partially purified preparation of Forsmann antibody was kindly supplied by Dr. Michael Edidin, and again no effect on the rate of aggregation of the neural retina cells was detected.

These results indicate that the effect of NRP on the neural retina cells is not the result of a nonspecific antibody-mediated agglutination reaction.

The Effect of NRP on Other Cells. Two types of experiments were conducted with NRP in the standard assay system. In the first, the effect of embryonic age of the neural retina cells was examined. Secondly, the effect of NRP on 5-day-old embryonic limb bud cells was tested.

The rate of aggregation (at 5 °C) of 10-day-old embryonic neural retina cells was compared with 8-day-old cells in the presence of increasing quantities of NRP (DEAE-cellulose fraction). As shown in Fig. 12, there was little or no difference in the reactivity of NRP with the two types of cell suspensions.

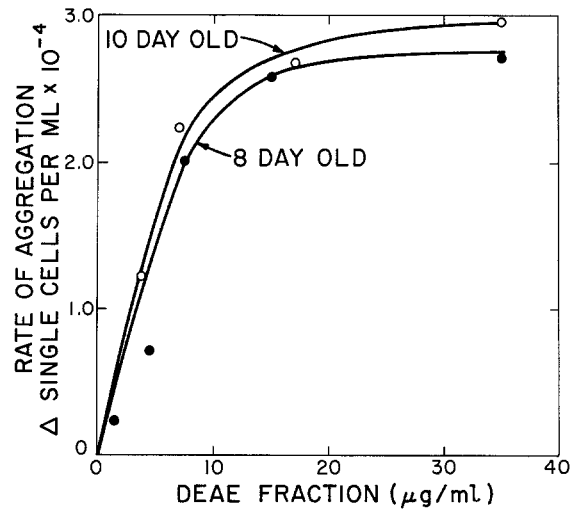

Fig. 12. The effect of increasing the amount of the concentrated DEAE-cellulose fraction on the rate of aggregation of 8-(•—•—•) and 10-(o—o—o) day-old chick neural retina cells. The initial density was 1.3×10^5 cells/ml, and duplicate flasks were removed at each concentration for both 8- and 10-day-old cells after 30 min at 5 °C. The rate of aggregation was calculated as described in the text

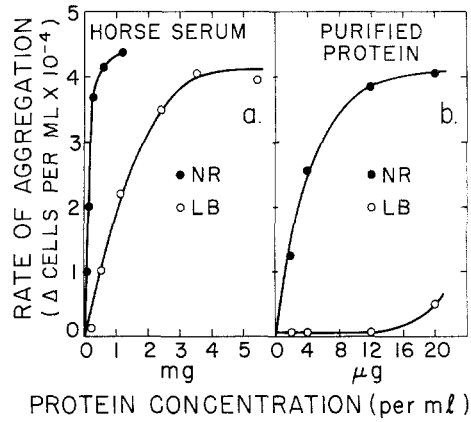

Fig. 13. The effect of (a) crude horse serum and (b) purified NRP (sucrose gradient fraction) on the rate of aggregation of 8-day-old chick neural retina cells and 5-day-old chick limb bud cells. Single cell preparations of both tissues were prepared as described in the text. The initial cell density for both tissues was 1.3×10^5 cells per ml. Duplicate flasks containing neural retina cells (•—•—•) or limb bud cells (○—○—○) were removed at each protein concentration after 30 min at 5 °C. The rate of aggregation was determined as described in the text

Horse serum was found to promote the aggregation of 5-day-old embryonic chicken limb bud cells, and the effect of increasing concentration of serum is shown in Fig. 13a. Examination of the figure shows that at the same initial cell densities, the horse serum at optimum concentrations promoted aggregation of the limb bud cells at approximately the same rate as neural retina cells. However, about six times more horse serum was required to obtain the same effect (for example, half maximum rate) with the limb bud in comparison to the retina cells. When the purified NRP was examined with both cell types, however (Fig. 13b), a much more dramatic difference was observed. The purified NRP showed no detectable effect on the limb bud cells until 20 μg/ml were used; even at this concentration, the rate with the limb bud suspension was barely detectable. The results suggest that horse serum contains at least two aggregation-promoting factors, one for neural retina and the other for limb bud cells. The factor that promotes the aggregation of limb bud cells was essentially removed during the purification of NRP. Attempts to isolate the limb bud factor are now in progress.

The Effects of Different Sera

Horse Sera. Different lots of horse sera were found to vary widely in their capacity to promote aggregation of neural retina cells. These results are summarized in Table 3. As can be seen, the differences vary within a

Table 3. *Activity of different horse sera*[a]

Horse serum (source)	Activity (Δ cells/ml $\times 10^{-4}$)
A (Gibco)	2.07
B (Gibco)	1.77
C (Gibco)	0.88
D (BioQuest)	0.29
E (BioQuest)	1.12

[a] In each case, 0.01 ml of sera was incubated with 1.3×10^5 cells/ml under standard conditions, for 30 min at 5 °C. The Δ was calculated as described in the text.

sevenfold range. Although the different lots of horse sera may actually vary in their NRP content, it is also possible that some of these sera contained inhibitors of NRP; this possibility was only tested with two sera, and gave negative results.

Chick and Calf Sera. Contrary to the results obtained with horse sera, two batches of chick and one of calf sera were tested for NRP, and were found to inhibit rather than to promote the aggregation of neural retina cells. The results are summarized in Table 4. As can be seen, the sera from these species inhibited the slow aggregation of neural retina cells at 5 °C normally observed in the control samples; the curves for these controls are shown in Figs. 1 and 7. Furthermore, the sera from chick and calf also inhibited the effect of NRP in the horse serum (Table 4). The addition of 0.10 ml of chick serum inhibited the stimulatory effect of 0.010 ml of horse serum to the extent of 55%, and when the experiment was conducted with

Table 4. *Inhibition of NRP activity of horse serum by chicken and calf sera*[a]

Conditions	Activity (Δ cells/ml $\times 10^{-4}$)
HH (control)	0.4
+ 10 µliters of horse serum	2.58
+ 100 µliters of calf serum	0
+ 100 µliters of chick serum	0.30
+ 100 µliters of calf + 10 µliters of horse sera	0.90
+ 100 µliters of chick + 10 µliters of horse sera	1.18

[a] Eight-day-old chick neural retina cells, at an initial density of 1.3×10^5 cells/ml, were incubated with the indicated sera under standard conditions for 30 min at 5 °C. In this case the activity is calculated by determining the net decrease in single cells [9] relative to those in medium containing calf serum (where no aggregation occurred).

calf in place of chick serum, a 75% inhibition in the rate of aggregation was observed. The nature of the inhibitory substance (or substances) in calf and chick sera is under study.

Discussion

In this paper and the preceding one [9], it has been shown that the rate of aggregation of 8-day-old neural retina cells is very slow at 5 °C. However, the rate is greatly enhanced if horse serum is added to the medium. The major purpose of this communication has been to describe the purification and some of the properties of the active material in horse serum. The purification has been simplified by slightly modifying the quantitative assay [9] for determining the rate of adhesion of single cells. Over a defined range of concentration, the rate was proportional to the horse serum concentration, thus facilitating the purification procedure. Using this assay, a protein was purified 48-fold from horse serum. Immunoelectrophoretic and ultracentrifugation studies of the most highly purified protein fractions showed a single component.

The purified NRP has a molecular weight of approximately 1.6×10^6 and an $S_{20\,w} = 22.4$. It does not migrate either in $7\frac{1}{2}\%$ acrylamide gel or on cellulose acetate electrophoresis. These characteristics indicate that NRP is a macroglobulin. Two observations suggest that this horse macroglobulin might be related to horse IgM. NRP and purified horse IgM cross-reacted with a goat anti-horse globulin fraction. Furthermore, NRP – like IgM – can be reduced to subunits by incubation with thiols. The reduced and alkylated subunits (of NRP) apparently retain the aggregating activity of the parent molecule, in sharp contrast to IgM which, on reduction and alkylation, loses its agglutinating (and precipitating) activity. NRP also differs from typical horse IgM on the basis of its sedimentation value which is considerably higher (22.4 compared to 19 S).

The following observations suggest that NRP is not simply a nonspecific cell agglutinating factor from horse serum: (a) NRP activity has, upon fractionation, always been found associated with a single fraction; it is unlikely that a single fraction would be capable of enhancing the aggregation of neural retina cells at 5 °C if it were nonspecific. (b) A purified horse IgM is inactive at concentrations 100-fold in excess of the optimum concentration of NRP. (c) Different horse sera vary widely in their activity. (d) A partially purified human Forsmann antibody is without effect on the rate of aggregation. (e) The rate of aggregation of 5-day-old limb bud cells

is unaffected by concentrations of NRP that cause a maximum rate of aggregation with neural retina cells.

Preliminary studies have shown that when cells are incubated with horse serum, the aggregating activity is lost from the medium after approximately 15 min. This result is currently interpreted to mean that the active material is adsorbed to the cell surface and exerts its effect there. Studies with ferritin-labeled antibody directed against purified NRP are currently underway to define this point rigorously.

Horse serum has been reported to have various effects on the aggregation of embryonic cells. Curtis and Greaves purified a protein inhibitory to aggregation and suggested that it could only be effective at low temperature (1 °C), because at higher temperatures the cells are able to degrade the inhibitor. Attempts to repeat these findings have been unsuccessful, although the negative results may be explained in several ways. In order to duplicate the results of Curtis and Greaves, it is imperative that the method of assay, the cell type, and the serum be identical. It has been mentioned that different sera show wide variations in NRP concentration. The possibility that some of these sera contain inhibitory species cannot be ruled out. Two of these sera have been purified extensively, and in these cases no inhibitory activity was detected. On the other hand, it has been possible to demonstrate inhibitory activity in two samples of chicken sera and in the one sample of calf sera tested to date. At high concentrations, these sera completely inhibit the aggregation of 8-day-old neural retina cells promoted by NRP at 5 °C. Furthermore the slow rate of aggregation that occurs in the absence of horse serum is prevented.

During the course of this investigation, it was found that the activity of NRP was destroyed when passed through a 0.22-μ Millipore filter. The reason for the inactivation is unclear, but the observation emphasizes how readily the activity could have gone undetected if sera were routinely Millipore-filtered before use.

Wyess and Burgess [11] have shown that the major cellular proliferation in the chick neural retina occurs by mitosis prior to the eighth day. Morphologically, all the cells are similar to each other and appear undifferentiated; subsequently, extensive differentiation occurs. These early observations have been substantiated recently by our own electron microscopic studies which indicate that 8-day-old neural retina cells are loosely arrayed except at the periphery of the tissue, where the beginning of morphological cell differentiation is just starting. The almost total lack of morphological differentiation does not preclude the possibility of extensive biochemical differentiation. These considerations led to an in-

vestigation of the effect of NRP on 10-day-old neural retina cells. It was found that the rates and degree of aggregation of 8- and 10-day-old neural retina cells (at 37 and 5 °C) were virtually equivalent. In biochemical terms, therefore, the 8-day-old cell surfaces must have areas of similarity, if not complete identity, with those of the 10-day-old cell. This conclusion is strengthened by the recent findings of Gershman [4] who compared the sorting-out behavior, electrophoretic mobility, rate of aggregation, and mean aggregate size of embryonic neural retina cells at different ages. Significant differences in some of these properties were observed between preparations obtained from 4- and 19-day-old embryos. Although retinas from 8- and 10-day-old embryos were not directly compared, the results clearly indicate that the properties listed above did not significantly change during this 2-day period. On the other hand, the apparent lack of response of 5-day-old chick limb bud cells to the purified NRP implies differences between the limb bud and neural retina cell surfaces. However, horse serum clearly contains material that promotes the rate of aggregation of limb bud cells at 5 °C. The nature of this material is currently under investigation.

It is not known whether the mechanism of action of NRP on neural retina cells at 5 °C is related to the normal process of adhesion at 37 °C. In any event, an understanding of the chemistry of this phenomenon should yield precise information concerning a surface constituent (or constituents) of the neural retina cell that is different from those on limb bud and perhaps other cells. A specific surface component is, of course, of great interest, particularly when it may play a role in intercellular adhesion.

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