AN ASSAY FOR INTERCELLULAR

ADHESIVE SPECIFICITY

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ABSTRACT

A modification of an assay for intercellular adhesive specificity is described. The method involves the collection of radioactively labeled cells by aggregates of the same (isotypic aggregates) or different (heterotypic aggregates) types of tissue and determination of the number of cells collected by liquid scintillation counting. The use of ^{32}P to label the tissues permitted a much more rapid estimation of cell collection than was obtained previously. With the use of chick embryo neural retina, liver, forebrain, pectoral muscle, and heart ventricle tissue, it was shown that isotypic was always greater than heterotypic collection. Labeled neural retina cell collection by neural retina aggregates was studied as a function of time, cell suspension density, aggregate diameter, temperature, and aggregate number. Neural retina aggregates were treated with certain enzymes in an attempt to determine whether specific changes on the surface of the aggregates would interfere with labeled neural retina cell collection. Of the various proteases and glycosidases tested, only β-galactosidase rendered the surface more nonspecific.

Intercellular adhesion apparently plays a key role in diverse biological phenomena (14). Studies from a number of laboratories have defined many biological parameters of the adhesive process: cells can adhere to glass or other nonbiological substrata (5), to each other in a specific or in a nonspecific manner (10), and can "sort out" when mixed with different cell types (13).

Neither the specific nor the nonspecific adhesive process has been explained in chemical terms, although we have recently shown that complex carbohydrates are involved in the intercellular adhesion of mouse teratoma cells (8). The present studies were aimed at defining the substance(s) on the surfaces of chicken embryonic neural retina cells required for the specific adhesion of these cells to each other. An obvious prerequisite for biochemical studies of this type is an assay method for adhesion. While a rapid and

reproducible quantitative method using the Coulter counter was developed in this laboratory (9), it could not discriminate between specific and nonspecific adhesion. A method which measures specific adhesion has been reported by Roth and Weston (11); labeled cells adhering to homologous and to heterologous collecting aggregates were counted by radioautographic methods. In the present studies, the specific adhesion assay of Roth and Weston was modified to permit more rapid estimation of the numbers of cells adhering to the aggregates. The method was then applied to studies on the specific intercellular adhesion of embryonic chicken neural retina cells. The available evidence suggests that terminal β-D-galactopyranosyl groups, presumably at the ends of the oligosaccharide chains of glycoproteins, mucins, and/or glycolipids, are involved in the specific adhesion of these cells.

MATERIALS

The following materials were obtained from the indicated commercial sources: HEPES buffer[1] (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), Calbiochem, Los Angeles, Calif.; chicken serum (heated at 56°C for 30 min), Medium 199, penicillin G, and streptomycin sulfate, Gibco Inc., Grand Island, New York; crude trypsin, 1:250, Difco Laboratories, Inc., Detroit, Mich.; crude collagenase, Worthington Biochemical Corp., Freehold., N. J.; Fungizone (amphotericin B), E. R. Squibb & Sons, New York; carrier-free phosphoric acid-^{32}P in 0.9% NaCl, New England Nuclear Corp., Boston, Mass.

All media were buffered to pH 7.2–7.4 with 0.01 M HEPES.

Nutrient medium, prepared aseptically, contained the following components (v/v): Medium 199, 60%; Hanks' basic salts solution, 25%; heat-inactivated chicken serum, 15%.

Labeling medium contained the following components: 2 mCi of carrier-free ^{32}P$_i$ in 0.1 ml of 0.9% NaCl; nutrient medium, 1.9 ml; penicillin, 200 units; streptomycin sulfate, 200 µg; and Fungizone, 1 µg.

Dissociating medium contained the following components: heat-inactivated chicken serum, 10% v/v; Ca^{++}- and Mg^{++}-free Hanks' basic salts solution, 90% v/v; Difco 1:250 trypsin, 0.1% w/v; and collagenase, 0.1% w/v. The medium was filter-sterilized before use.

METHODS AND RESULTS

Assay for Intercellular Adhesive Specificity

The assay used in these studies, which is a modification of that reported by Roth and Weston (11), is designed to compare intercellular adhesion between cells of the same type (isotypic), with adhesion between cells of different types (heterotypic). In the original assay, "collecting aggregates" were circulated in a thymidine-^{3}H–labeled cell suspension. The aggregates were composed either of cells of the same type as those which were labeled, or of a different type. The number of labeled cells which adhered to the collecting aggregates was determined by radioautographic methods. When eight different types of tissue from mouse and chick embryos were tested, isotypic adhesions were found to be greatly favored in nearly every case. However, the method

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[1] *Abbreviations used in this paper:* DNP, dinitrophenol HEPES buffer, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; POPOP, 1,4 bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole.

was tedious, requiring several weeks for analysis of each experiment.

In the present studies, the cells were labeled to a much higher degree by incubating whole tissue in phosphoric acid-^{32}P–containing media. Two to three intact livers or neural retinas were removed aseptically from 8-day old White Leghorn chicken embryos, washed briefly in nutrient medium, and incubated in the labeling medium for 16–20 hr at 37°C on a gyratory water bath shaker (New Brunswick Scientific Company, New Brunswick, N. J., model G-76) at 75 rpm. After incubation, the tissues were removed from the medium and washed six times in 10-ml portions of glucose-free Hanks' solution. Labeling was more efficient when the labeling medium had been used at least once; therefore the medium was first incubated with undissociated neural retina as described, the tissue was discarded, and the medium was then employed for labeling fresh tissue. The labeling medium could be subsequently used repeatedly and efficiently for periods up to 2 wk. The labeled tissues were dissociated by incubating them for 10 min in 4 ml of dissociating medium at 37°C. After this incubation, the tissue fragments were aspirated several times through a small-bore Pasteur pipette, and once again incubated for 10 min in the dissociating medium. After further aspiration to complete the dissociation of the cells, the suspension was centrifuged at 200 g for 4 min at 4°C, and the cells were washed twice with 4 ml of either nutrient medium or glucose-free Hanks' solution, and maintained at 0°C until used (never more than 15 min). In general, this procedure gave between 1 and 4 cpm per cell.

Unlabeled aggregates were prepared by dissociation of the appropriate tissue as described above, and subsequent reaggregation on a gyratory shaker (6). The unlabeled single cell suspensions were diluted with nutrient medium to a final concentration of 2.2 × 10^6 cells/ml, and portions containing 4 ml of suspension were placed in 25-ml DeLong culture flasks and rotated at 75 rpm, 37°C, for 16–20 hr to promote aggregation. At this time, aggregates with diameters of 0.8 ± 0.1 mm were selected under a dissecting microscope with a stage micrometer.

The preparation of labeled single cell suspensions and unlabeled aggregates was generally conducted at the same time; it is important to note that while 8-day old chicken embryonic

tissue was employed for these studies, the tissues had been incubated for an additional day during the labeling experiments, and may perhaps be equivalent to 9-day old rather than 8-day old cells in terms of their development.

Collection of labeled single cells by unlabeled aggregates was initiated by adding a specified number of aggregates to 4 ml of single cell suspension (in either nutrient medium or glucose-free Hanks' solution) in a 25 ml DeLong culture flask, and placing the mixture on a reciprocating water-bath shaker; the shaker operated at 60 oscillations/min, with a 1.5-inch stroke per oscillation. Specific conditions, such as time of incubation, cell density, and number of aggregates, are given for each experiment.

After the aggregates had been circulated in the labeled cell suspensions for the specified time, they were removed from each DeLong flask with a Pasteur pipette and washed by dropping them into a 500 ml separatory funnel containing glucose-free Hanks' solution. The passage of the aggregates from the top to the bottom of the funnel separated them from most of the nonadhering cells. The stopcock (Teflon, 2 mm bore) was then opened and the aggregates were quickly collected in a Petri dish containing 30 ml of glucose-free Hanks' solution. The aggregates were then deposited directly on Whatman No. 1 chromatography paper (1 × 2 inches) and dried at room temperature for 1 hr. The papers, each containing all the aggregates from a single flask, were then counted in a toluene solution containing 4 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4 bis[2-(5-phenyloxazolyl)]benzene (POPOP)/l, in a Packard Tri-Carb liquid scintillation spectrometer.

The number of counts per minute per cell was estimated in each experiment by determining the cell density with a hemocytometer and by counting 0.1 ml of the labeled suspension by liquid scintillation techniques. Viability was determined by dye exclusion criteria with trypan blue. From 5 to 10% of freshly dissociated, unlabeled cells did not exclude the dye, whereas the number of stained cells in the dissociated, labeled cells ranged from 15 to 20%.

In the original assay procedure (11), the label which appeared in the aggregates at the end of each experiment was shown by radioautographic methods to result from the adhesion of labeled single cells to the aggregates. In the present case,

where such techniques were not employed, it was possible that some radioactivity in the aggregates could have been obtained from label leaked into the medium from the labeled single cells, and was *not* the result of cell adhesion to the aggregate. In fact, a large number of experiments indicated that between 10 and 30% of the label was lost to the medium from the single cells over a 2 hr incubation period under standard assay conditions. The following control was therefore employed for each experiment: Labeled cell suspensions were shaken without aggregates for 2 hr under standard conditions. The cells were then removed by centrifugation, and the labeled supernatant was incubated for an additional 2 hr under standard conditions with aggregates of the same type as employed in each experiment. The aggregates were then collected and counted as described above. In all cases, the control aggregates exhibited less than 30 cpm per aggregate (above background) and these values were subtracted from the experimental values.

LABELING OF LIVER AND NEURAL RETINA CELLS BY DIFFERENT SUBSTRATES: A large number of isotopic substances were tested to determine optimal conditions for labeling the cells used for collection. The results obtained with the different compounds are shown in Table I. ^{32}P consistently gave higher labeling and was routinely used.

VARIABILITY OF THE ASSAY: The speed and convenience of the present method provide the major advantages over the radioautographic procedure previously described (11), and permit the analysis of a large number of samples. There was considerable variability in replicate flasks, so that all of the experimental values in each experiment are given in the tables, as well as their mean values. The tables show that the assay method may be considered semiquantitative, but provides results sufficiently quantitative and reproducible to draw certain conclusions concerning the nature of specific intercellular adhesion.

Specificity and Kinetic Parameters

Fig. 1 shows the extent of collection of labeled liver cells by 8-day old liver and neural retina cell aggregates as a function of time. Collection was carried out in nutrient medium at 37°C with three aggregates per flask. After a lag of about 2 hr, isotypic collection begins and proceeds at a rate far greater than heterotypic collection which

remains minimal for at least 5 hr. The results of the reciprocal experiment are shown in Fig. 2. The lag in isotypic neural retina cell collection in nutrient medium at 37°C, when observed, is clearly shorter than with liver, although heterotypic collection again remains low. Figs. 3 and 4 show neural retina isotypic collection, again in nutrient medium at 37°C, as functions of cell suspension density and aggregate diameter. Col-

lection increases with the suspension density up to 8×10^5 cells/ml. The correlation with aggregate diameter is given in Fig. 4; values are given for each of duplicate flasks with four aggregates per flask. Aggregates approximately 0.8 mm in diameter were routinely used, since in this part of the curve the results would be less affected by slight variations in aggregate size.

STUDIES ON THE LAG: In five different ex-

TABLE I

Degree of Labeling Attained with Selected Radioactive Compounds

| Specific activity of compounds | Total per incubation* | Time of incubation | Degree of labeling | |
			Liver	Neural retina
cpm/μmole	*cpm*	*hr*	*cpm/cell*	
HCO_3^--^{14}C, 4×10^8	5×10^7	2	0.05	0.03
" "	2×10^7	15	0.10	—
" "	10^9	5	0.207	0.093
" "	10^9	24	0.122	——
Thymidine-^3H, 10^{10}	5×10^7	29	0.05	0.02
Glucose-^{14}C, 6×10^5	1.2×10^7	1	—	0.0021
" "	1.2×10^7	3	—	0.0043
Sugars-^{14}C, 10^8‡	2.5×10^8	3	—	0.033
" "	2.5×10^8	5	0.08	0.058
" "	2.5×10^8	10	—	0.078
" "	10^9	5	0.211	0.094
" "	4×10^9	5	—	0.18
" "	4×10^9	24	—	0.422
" "	4×10^9	30	—	0.380
" "	4×10^9	30§	—	0.374
Sugars-^{14}C, 10^8 plus HCO_3^--^{14}C, 4×10^8	5×10^9	7	0.88	—
" "	5×10^9	7§	1.06	—
" "	5×10^9	24	0.98	0.36
" "	5×10^9	24	0.56	0.35
" "	5×10^9	26	1.76	0.402
Pyruvate-^{14}C, 2×10^7	2×10^8	24	<0.1	<0.1
Sugars-^{14}C, 10^8 plus pyruvate-^{14}C, 2×10^7	$\sim 4 \times 10^9$	24	1.41	0.410
Phosphoric acid-P^{32}, carrier-free	10^9	16	~ 2.0	~ 2.0

* The labeled compounds were substituted for phosphoric acid-P^{32} using the procedures described in the text.

‡ Sugars-^{14}C are obtained by alcohol extraction of tobacco leaves grown in the presence of $^{14}CO_2$ (2×10^8 cpm per μmole).

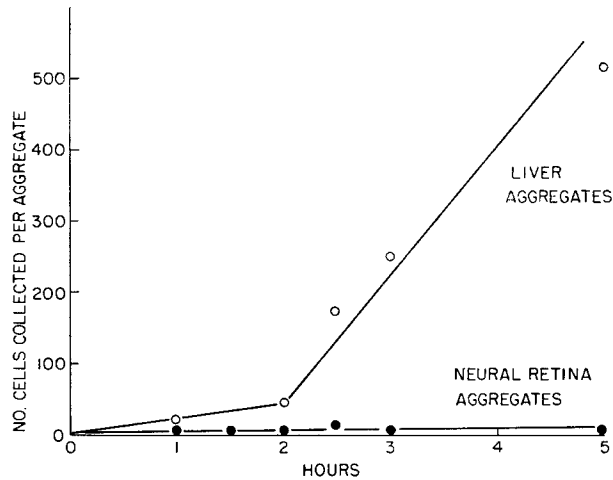§ In the presence of 1 unit of insulin/ml of incubation medium.

FIGURE 1 Collection of ^{32}P-labeled liver cells by liver and neural retina cell aggregates as a function of time. Cell concentration was 1.5 × 10⁵ cells/ml (1.1 cpm per cell) with three aggregates per flask in nutrient medium, and the temperature was 37°C.
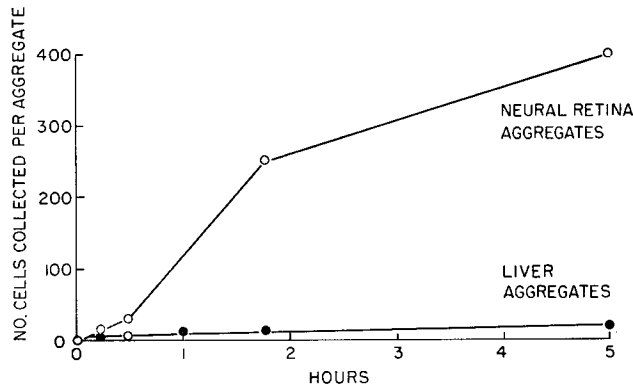

FIGURE 2 Collection of ^{32}P-labeled neural retina cells by neural retina and liver aggregates as a function of time. Cell concentration was 1 × 10⁵ cells/ml (1.0 cpm per cell) with three aggregates per flask in nutrient medium, and the temperature was 37°C.

periments, the lag in isotypic neural retina cell collection at 37°C varied between 0 and 15 min. Methods were examined for consistently extending this period. It was found that at 28°C the lag in cell collection was extended to 25–30 min and was reproduced in six different experiments. Fig. 5 illustrates the results of a typical experiment showing neural retina collection by neural retina aggregates at 28°C. Each point is the mean of four flasks with five aggregates per flask. The range of the four values is also shown. Unless specified otherwise, subsequent experiments were conducted with isotypic collection of neural retina cells at 28°C.

COLLECTION BY DIFFERENT AGGREGATE TYPES: In order to test the specificity for neural retina cell collection with aggregates other than liver, an experiment was carried out in which collection by equal-sized aggregates of three different types was compared with neural retina isotypic collection. Table II shows that, at best, the heterotypic aggregates collect less than 25% of the labeled neural retina cells collected by neural retina aggregates.

FACTORS LIMITING COLLECTION: During the few hours of incubation relatively small numbers of cells of the total population adhered to the aggregates. Thus, it was possible that specific
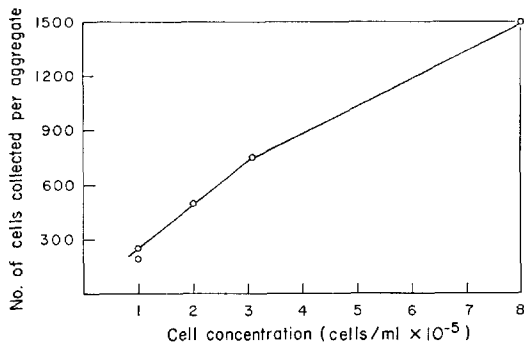
FIGURE 3 Collection of ^{32}P-labeled neural retina cells by aggregates of neural retina as a function of density of cell suspension. Collection was carried out for 90 min in nutrient medium at 37°C with 0.5 cpm per cell in the presence of aggregates and varying numbers of cells as indicated.
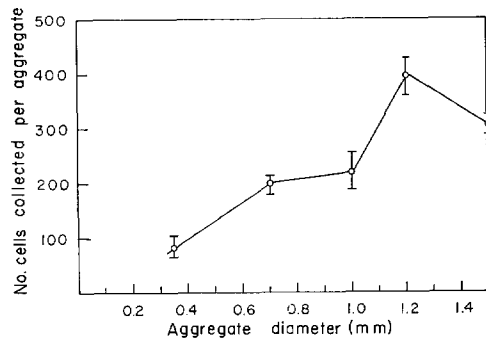

FIGURE 4 Isotypic collection of ^{32}P-labeled neural retina cells as a function of the diameter of the aggregates. Collection was carried out for 90 min in nutrient medium with 1×10^5 cells/ml (0.5 cpm per cell) at 37°C in the presence of four aggregates of each size in each flask. Each point is calculated from a scintillation count of all the aggregates in one flask.

adhesion was the special property of a small fraction of the total single cell population. To answer this question, adhesion studies were conducted in the presence of varying numbers of collecting aggregates. Four separate flasks were prepared containing 4-ml portions of a labeled neural retina cell suspension of 3×10^5 cells/ml of nutrient medium. Four neural retina aggregates were deposited into each of three of the flasks and 80 aggregates were deposited into the fourth. Collection was conducted at 28°C. The four aggregates from each of the first three flasks were removed at 30, 60, and 90 min, respectively. From the fourth flask, containing 80 aggregates,

four aggregates were taken at 30 min, four more at 60 min, and two groups of four at 90 min. The remaining 64 were all removed at 150 min and were washed, dried, and counted in four groups of 13 aggregates each and one group of 12. The results clearly show that increasing the number of collecting aggregates from 4 to 80 per flask affects the rate of the process (Fig. 6), being slower where the number of collecting aggregates is very large, but that ultimately the number of
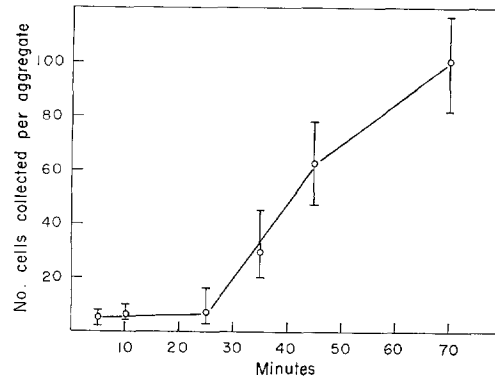

FIGURE 5 Collection of ^{32}P-labeled neural retina cells by neural retina aggregates at 28°C. The curve represents the results from an experiment using 10^5 cells/ml (1.3 cpm per cell). Each bar includes the range of four points, each of which represents one flask (five aggregates per flask).

TABLE II

Collection of Labeled Neural Retina Cells by Aggregates of Different Types

Collection was carried out in glucose-free Hanks' solution for 2 hr at 28°C with 1.3×10^5 labeled neural retina cells/ml at 3.3 cpm per cell.

Type of aggregate		Mean No. of cells collected per aggregate	Cells collected as % of No. collected per neural retina aggregate
	days		
Neural retina,	8	701 (545, 1080, 480)*	100
Forebrain,	8	163 (256, 70)	23
Pectoral muscle,	12	143	20
Heart ventricle,	8	22	3

* Numbers in parentheses indicate the replicate values obtained with different flasks.

single cells that adhere to each aggregate is about the same. Further, and more important, after 150 min, about 5% of the cells in the flask were collected by the 80 aggregates. It appears that the limiting factor in cell collection is the low frequency of encounter between cell and aggregate or a low probability of adhesion after an encounter occurs, rather than the number of cells capable of adhering to the aggregate.

EFFECTS OF METABOLIC INHIBITORS: Table III shows that azide, cyanide, dinitrophenol, and low temperatures have inhibitory effects on isotypic neural retina cell collection while fluoride has little, if any, effect at the concentrations used. In two additional experiments under similar conditions, puromycin and cycloheximide, both at concentrations as high as 10 μg/ml, had no effect on collection up to 80 min (not shown).

ENZYME TREATMENTS: It is assumed that the lag in collection of labeled cells by aggregates is the result of the time required for regeneration of specificity-conferring components on the dissociated cell surfaces. Adhesion to aggregates cannot take place with cells lacking these components, so that, even after the lag period, only cells with matching components can adhere to the aggregates. Treatment with known enzymes which could make an aggregate surface more nonspecific would give information on the nature of the
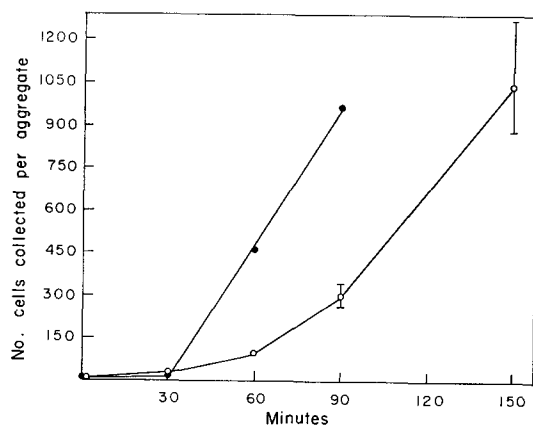

FIGURE 6   Effect of the number of aggregates present on the isotypic collection of ^{32}P-labeled neural retina cells. Details are given in the text. In this experiment, no controls were done for uptake of noncellular radioactivity, and the values presented are calculated from the actual count. ●——●, four aggregates per flask; O——O, 80 aggregates per flask. The bar at the 150 min time point represents the range of five flasks.

TABLE III

Effects of Metabolic Inhibitors on Isotypic Neural Retina Collection

In Experiment 1, 2.2 × 10⁵ cells/ml of nutrient medium (3.2 cpm per cell) were incubated in the presence of various concentrations of inhibitors for 80 min at 28°C. For Experiment 2, the medium was glucose-free Hanks' and the cell density was 1.0 × 10⁵ cells/ml (4.0 cpm per cell). Incubation was conducted for 60 min at 28°C in the presence of the indicated inhibitors. Both experiments were also performed at 0°C without added inhibitors.

Inhibitor	Mean No. of cells per aggregate	% of control
μmoles/ml		
Experiment 1		
None	244 (202, 286)*	100
NaF, 2	212 (144, 280)	87
NaN₃, 1	125 (64, 187)	50
NaN₃, 2	74 (55, 94)	30
NaN₃, 3	31 (14, 47)	13
NaCN, 2	38 (25, 51)	15
None, 0°C	6	2
Experiment 2		
None	200 (209, 178, 213)	100
NaN₃, 2	117 (135, 107, 110)	58
NaCN, 2	81	41
Dinitrophenol, 2	102	51
None, 0°C	19	9

* Numbers in parentheses indicate replicate values.

components being attacked. Accordingly, aggregates were treated with several different enzyme preparations.

Neural retina aggregates were incubated in Hanks' solution with 0.1% crude trypsin (Difco Laboratories, Inc., 1:250) and/or 0.1% crude collagenase (Worthington Biochemical Corp.) at 37°C for 25 min. These aggregates were then washed in two 30-ml volumes of Hanks' salt solution. Control aggregates were incubated in Hanks' salt solution at 37°C and washed similarly. There was no change in the number of neural retina cells collected in 25 min at 28°C in the experimental as compared with the control aggregates.

In the absence of an effect with proteases, aggregates were treated, as above, with a crude extract of *Diplococcus pneumoniae* type I supernatant (2) containing many glycosidases. The experi-

mental aggregates collected more than five times the number of neural retina cells collected by control aggregates in 25 min at 28°C. A partially purified β-galactosidase preparation from this supernatant was able to duplicate this effect. The enzyme was purified using p-nitrophenyl β-D-galactoside as a substrate according to methods described by Hughes and Jeanloz (2). This enzyme is active with large as well as low molecular-weight substrates. The resulting Diplococcal β-galactosidase preparation had no detectable

protease activity (no color released after 100 units were incubated overnight with Azocoll at 37°C), no detectable β-N-acetylglucosaminidase activity against the corresponding p-nitrophenyl derivative, and no detectable sialidase activity using sialyllactose as substrate. The effect of this preparation on neural retina aggregates is shown in Table IV, experiment 1.

If the increase in cell collection during the lag is due, in fact, to β-galactosidase and not to a contaminating activity, then another β-galactosid-

TABLE IV

Effect of Treatment of Neural Retina Aggregates with β-D-Galactosidases from D. pneumoniae and C. perfringens on Neural Retina Cell Collection

Experiment 1, aggregates were first incubated with the indicated quantities of β-D-galactosidase from D. pneumoniae in a total volume of 4 ml on a gyratory water bath shaker at 75 rpm for 15 min at 37°C. After appropriate washing (see text), collection was carried out with three aggregates per flask and 1×10^5 cells/ml (1.2 cpm per cell) for 25 min at 28°C.

Experiment 2, aggregates were treated in a total volume of 4 ml on a gyratory water bath shaker at 75 rpm with the indicated quantites of β-D-galactosidase from C. perfringens, for 15 min at 37°C. After appropriate washing (see text), collection was carried out with four aggregates per flask and 1×10^5 cells/ml (4.8 cpm per cell) for 25 min at 28°C.

Experiment 3, aggregates were treated in a total volume of 4 ml on a gyratory water bath shaker at 75 rpm for 15 min at 37°C with the specified quantities of the indicated enzymes. Samples were taken from the actual treatment flask for assay against the synthetic substrate. Collection was carried out with four aggregates per flask and 10^5 cells/ml (2.5 cpm per cell) at 28°C for 25 min.

Previous treatment of aggregates	Mean No. of cells collected per aggregate
Experiment 1, *D. pneumoniae* β-galactosidase	
Glucose-free Hanks' medium	7 (10, 3)*
+12.5 units/ml of β-galactosidase	19 (14, 23)
+25 units/ml of β-galactosidase	21 (17, 24)
+100 units/ml of β-galactosidase	43 (14, 71)
+100 units/ml of heated β-galactosidase (56°C, 3 min)	8 (7, 8)
Experiment 2, *C. perfringens* β-galactosidase	
Glucose-free Hanks' medium	5 (4, 6)
+20 units/ml of β-galactosidase	7 (3, 5, 14)
+80 units/ml of β-galactosidase	16 (7, 18, 23)
+200 units/ml of β-galactosidase	24 (16, 15, 37)
+200 units/ml of heated β-galactosidase (100°C, 15 min)	13 (12, 8, 18)
Experiment 3, *Diplococcal* and *Clostridial* β-galactosidases	
Glucose-free Hanks' medium	5 (3, 6)
+61 units/ml of *Diplococcal* enzyme	15 (11, 16, 17)
+81 units/ml of *Clostridial* enzyme	13 (16, 10)
+61 units/ml of *Diplococcal*, and +81 units/ml of *Clostridial* enzyme‡	23 (15, 21, 32)
+61 units/ml of *Diplococcal* and +81 units/ml of *Clostridial* enzyme in 10 mM D-galactal§	13 (10, 17)

* Numbers in parentheses indicate replicate values.

‡ Assay with p-nitrophenyl β-D-galactopyranoside gave 138 units/ml. A unit of activity is defined as 0.01 absorbance unit at 400 mμ as described by Hughes and Jeanloz (2).

§ Assay with p-nitrophenyl β-D-galactopyranoside gave 22 units/ml.

ase isolated from a different source should give a similar result. Experiment 2 in Table IV shows the results obtained when aggregates were treated with β-D-galactosidase purified from a *Clostridium perfringens* supernatant (1). Although this preparation had β-*N*-acetylglucosaminidase activity, β-galactosidase-free β-*N*-acetylglucosaminidase from the same organism had no effect on isotypic neural retina collection. (Both of the *Clostridial* enzyme preparations were kind gifts of Mr. Stefan Chipowsky.) The combination of *Clostridial* and *Diplococcal* β-galactosidases (Table IV, experiment 3) had an additive effect on neural retina cell collection and this effect was sensitive to the β-galactosidase inhibitor, D-galactal (3), although the extent of inhibition of the enzymes using the *p*-nitrophenyl β-D-galactopyranoside substrate was much greater than the inhibition of the enzymatic activities in the cell collecting assays.

Table IV presents the results of three of eight similar experiments. While considerable variability is apparent in each experiment, all eight experiments showed essentially the same results.

If β-galactosidase treatment does render neural retina aggregate surfaces less specific, then treated aggregates should collect more heterotypic cells than untreated neural retina aggregates. Table V shows that, in fact, this is the case. Treatment with both β-galactosidases, however, had no effect on the collection of liver or neural retina cells by liver aggregates.

EFFECT OF AZIDE AND β-GALACTOSIDASE ON SPECIFIC ADHESION: When untreated

TABLE V

Effect of Clostridial β-Galactosidase-Treated Neural Retina Aggregates on Collection of Labeled Liver Cells

Aggregates were first treated in a total volume of 4 ml on a gyratory water bath shaker at 75 rpm for 30 min at 37°C. Collection was carried out with five neural retina aggregates per flask with cell concentration of 2 × 10^5/ ml (2.1 cpm per cell) for 30 min at 28°C.

Previous treatment of neural retina aggregates	Mean No. of liver cells collected per aggregate
Glucose-free Hanks' medium	6 (7, 5, 5)*
Glucose-free Hanks' medium + 100 units/ml of enzyme	15 (24, 6, 12, 18)

* Numbers in parentheses indicate replicate values.

neural retina aggregates are used for collecting isotypic cells, the cells require 20–30 min before they can adhere to the collecting aggregates. However, when the aggregates are pretreated with β-galactosidase, they begin to collect cells before the 20–30 min lag period otherwise observed. Since azide prevented collection of cells by untreated aggregates, the effect of azide was tested on the collection of freshly dissociated cells by galactosidase-treated and control aggregates.

Fig. 7 shows that untreated aggregates can collect cells in the presence of azide if the freshly dissociated cells are first incubated without azide for 30 min. If the cells are exposed to azide immediately after dissociation, they can adhere to β-galactosidase–treated aggregates (treated for 20 min at 37°C) more efficiently than to untreated aggregates.

DISCUSSION

The assay presented above is modified from the original procedure of Roth and Weston (11). In this method, a suspension of labeled single cells is shaken with "collecting aggregates," which were of the same or different cell types as the single cell suspension. We assume that difference in collection represents differences in adhesive specificity. In the original procedure (11), the estimation of the cells which adhered to the aggregates was performed by lengthy radioautographic techniques requiring at least 25 days. In the present procedure, an experiment can be conducted in 1 day.

Since cells exhibiting much higher concentrations of radioactive label were required for the present method, various isotopes were tested, the most effective being inorganic phosphate-[32]P. The label was incorporated into incubated neural retina tissue to the extent of 0.5–4.0 cpm per cell. With this isotope a significant quantity of label was lost from the cells during the aggregation assay and there was a possibility that the labeled material appearing in the aggregation medium might be absorbed by the collecting aggregates. However, this could be compensated for by appropriate experimental controls. There are certain other problems inherent in the method which have not yet been satisfactorily resolved: (*a*) In the Coulter counter procedure (9), the rate of adhesion of cells could be measured to the point where differences on the order of 10–15% were detectable. However, specificity could not
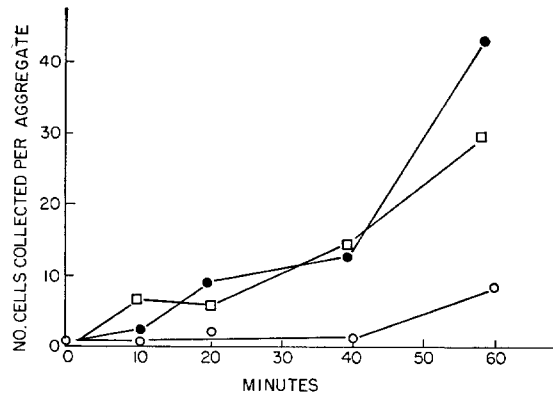
FIGURE 7 Effect of azide and β-galactosidase on specific adhesion. Collection was carried out with a ^{32}P-labeled neural retina suspension of 1 × 10^5 cells/ml (1.7 cpm per cell) in the presence of 4 μmoles/ml of NaN$_3$. Three systems were used: O——O, neural retina suspension with untreated aggregates; □——□, neural retina suspension incubated at 28°C in glucose-free Hanks' solution for 30 min without azide before addition to flask containing untreated aggregates; and ●——●, neural retina suspension with aggregates which had been incubated with 150 units/ml of Diplococcal β-galactosidase in glucose-free Hanks' for 20 min at 37°C before collection.

be distinguished by this method. The present procedure can demonstrate adhesive specificity, in spite of the fact that replicate samples in different flasks occasionally show variations of as much as 100%, although the usual range is 25%. We believe that the major cause is that small groups of cells (as well as single cells) may adhere to the collecting aggregates. (b) Another inherent difficulty in this method lies in the small numbers of cells which are collected by the aggregates. Any tissue which is dissociated to a suspension of single cells contains a variety of cell types. Do the single cells collected by the aggregates represent a major or a minor fraction of the cell types in the total population? This question cannot be answered with certainty, but the experiment described in Fig. 6 shows that when a large number of collecting aggregates is used per flask, about 5% of the total single cell population adhere to the aggregates. On the other hand, the rate of adhesion per aggregate in the presence of large numbers of collecting aggregates was slower than the rate in the presence of small numbers of collecting aggregates. This could be due to the gradual acquisition of specificity-conferring components by all the cells or to the fact that a special class of cells was being collected.

In spite of these limitations, the procedure clearly manifests an adhesive recognition and appears to be a useful method for measuring this important process.

Results of studies on the collection of labeled

neural retina cells by neural retina aggregates indicate that collection begins after a variable lag at 37°C and after a definite 25–30 min lag at 28°C. They show, as well, that collection is specific; liver, forebrain, muscle, and heart aggregates collect fewer neural retina cells than do neural retina aggregates under the same conditions. It was also demonstrated that neural retina isotypic collection is sensitive to several metabolic inhibitors.

In contrast to these results, other investigators working with simple reaggregation of neural retina cells after dissociation have shown that (a) there is no detectable lag in single cell disappearance at 37°C or 25°C (4, 9), (b) after dissociation, neural retina cells will adhere to several other cell types nonspecifically (6, 12), and (c) aggregation of neural retina cells is not sensitive to azide, cyanide, or dinitrophenol (DNP) for at least the first 2 hr after dissociation (9).

The simplest explanation for these differences is that cells dissociated from each other by treatment with crude trypsin lack surface components required for selective adhesion. These cells are capable of nonspecific adhesion to each other, but adhere poorly to collecting aggregates which contain regenerated or "complete" cell surfaces. During the lag period, the single cells regenerate the necessary components which permit them to adhere to the intact collecting aggregates. To test this hypothesis, the surfaces of the cells in the collecting aggregates were modified by treatment

with specific enzymes. It was found that neural retina aggregates treated with two partially purified β-galactosidases collected more liver cells than did untreated aggregates; in other words, treatment of the neural retina aggregates with β-galactosidase made them less specific. Furthermore, if single neural retina cells are poisoned with azide, they are unable to regenerate the surface components required for specific adhesion. Such cells adhered more rapidly to β-galactosidase-treated aggregates than they did to untreated aggregates, again indicating that β-galactosidase treatment of the neural retina aggregates decreased their specificity. Finally, when single cells were permitted to recover from the trypsin treatment by incubation for 30 min at 28°C in the absence of azide, and were then suspended in a medium containing azide, the treated aggregates collected fewer cells than did the untreated aggregates.

The results of these experiments showed that treatment with β-galactosidase changed the adhesive properties of the collecting aggregates, making them more, but not totally, nonspecific. These results might be expected since it appears reasonable to suppose that many sites on the cell surface are responsible for specific adhesion, and not all of them are sensitive to β-galactosidase. Thus, β-galactosidase treatment would probably yield a "mixed" cell surface with respect to adhesive properties rather than a complete change. Nevertheless, it appears reasonable to conclude that terminal β-galactosyl residues on the surfaces of neural retina cells are at least partly responsible for the adhesive selectivity in these cells.

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