dispersal on Wegener's original reconstruction of Cretaceous paleogeography, in which North America, South America, Antarctica, and Australia are assumed to have formed a single connected land mass after South America and Africa had already been separated by the embryonic Atlantic Ocean; this arrangement and the implied dispersal routes are not conformable with modern geophysical evidence (*4*, pp. 4946–4947).

Cox's (*20*) interpretation of the early dispersal of marsupials is largely accepted and incorporated into the hypothesis presented here. Martin's (*21*) suggestion that marsupials reached Australia and the Americas by dispersal from a hypothetical land mass isolated in the South Pacific Ocean fails, as noted by Cox, to explain how the ancestral marsupial stock reached this isolated center of dispersal. Clemens' (*14*, p. 381) recent suggestion that marsupials may have spread from South America to Antarctica and Australia across a Cretaceous or early Tertiary water gap seems implausible, since it invokes chance to account for the failure of South American placentals to accompany marsupials on this over-water migration and it implies that the occurrence of monotremes and marsupials in the Australian region also is the result of chance.

The hypothesis presented here has several obvious implications that may provide tests of its validity. First, this hypothesis should be applicable to interpretation of later mammalian zoogeography, after the separation of Africa and Madagascar. It should help to explain the Tertiary mammalian faunal history of North America after its separation from Europe and during its subsequent union with Asia via the Bering bridge and with South America via the Isthmus of Panama. It should also apply to the mammalian faunal history of India during the long isolation of that subcontinent and after its union with Asia along the Himalayan suture. Second, the hypothesis developed here for mammals should be applicable to other vertebrates, to invertebrates, and to plants. It should help to explain the distribution of a number of Southern Hemisphere nonmammalian vertebrates, such as lungfishes, osteoglossid fishes, leiopelmatid and hylid frogs, side-necked turtles, *Sphenodon*, iguanid lizards, booid and elapid snakes, ratite birds, and tinamous. Lynch (*22*, p. 218) has already interpreted the distribution of leptodactyloid frogs in a way that generally agrees with the hypothesis presented here. Finally, this hypothesis implies certain past distributions not yet documented by the fossil record. Marsupials should be anticipated in the African Cretaceous, as postulated by Cox (*20*, p. 770), and prototherians should be expected in the African Jurassic or Cretaceous. Similarly, prototherians and metatherians should be present in the Mesozoic and early Tertiary of India. Prototherians and metatherians also should be anticipated in Antarctica above the Lower Triassic beds that recently have yielded labyrinthodont amphibians, thecodont reptiles, and therapsid reptiles (*23*).

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29. I thank H. Dybas and P. Hershkovitz, colleagues at the Field Museum of Natural History, with whom I have had numerous discussions of these ideas. I also thank L. B. Radinsky and L. Van Valen, University of Chicago, who read and commented on an early version of the manuscript.

18 August 1971; revised 1 October 1971 ■

Cell Aggregation: Role of Acid Mucopolysaccharides

Abstract. *Factors that induce cell aggregation are released by several types of chick embryo and mammalian cell cultures. These aggregation factors are also present in some serums. The factors in each of the preparations tested were inactivated by treatment with bovine testicular hyaluronidase. Conversely, hyaluronic acid promoted aggregation of only those cells that were aggregated by media containing the factors. These factors appear to be acid mucopolysaccharides, with hyaluronic acid being a major component.*

Dissociated cells from sponges (*1*), from embryos (*2*), and from some tissue cultures (*3*), are able to reassociate to form multicellular structures, or aggregates. From these cells, substances with an aggregation-promoting activity (aggregation factors, or AF's) have been obtained by various methods (*4*, *5*). Some of these AF's apparently exert their effects by interaction with components at the cell surface. For example, AF's obtained after chemical dissociation of sponges or by spontaneous cellular release in monolayer cultures enhance the aggregation of the corresponding mechanically dissociated cells at 4°C (*6*, *7*). On the basis of preliminary evidence, it has been suggested that the sponge AF's, large molecules with a definable ultrastructure, are composed of glycoproteins (*8*). We now report that the AF's released from mammalian cells and from chick embryo neuroretinal and liver cells in culture and the AF's present in serums appear to be acid mucopolysaccharides (MPS).

To obtain the AF's released from

cells, monolayer cultures in milk dilution bottles were washed three times with phosphate-buffered saline (PBS) and incubated at 37°C in 10 ml of Eagle's basal medium with double-strength amino acids (2× BME) without serum (*7, 9*). After 24 hours the media were collected and centrifuged, first at 300*g* for 10 minutes and then at 10,000*g* for 30 minutes. The supernatants containing the AF's were assayed quantitatively for aggregation-promoting activity on cells from the mouse lymphoblastic cell line P388 (*10*) by the gyratory shaker method (*11*). The P388 cells themselves did not release any AF, but all other cells released from 200 to 30,000 units of AF's per milliliter of medium. The average titers for each cell type are given in Table 1. The supernatants were tested for aggregation activity on homologous or different cells; no specificity with respect to cell type or species was observed (*12*). Serums from various sources (fetal calf, calf, horse, and chicken) were assayed in the same manner as the supernatants from the cells and they also had an aggregation-inducing effect on the P388 cells (*13*).

Supernatants were submitted to various treatments and titered on P388 cells. No aggregation activity was lost by heating the liquid at 100°C for 15 minutes or by treatment with deoxyribonuclease, ribonuclease, collagenase, trypsin, pronase, or neuraminidase (*14*). However, treatment for 24 hours at 4°C with periodate (0.01*M*, *p*H 5.5) completely inactivated the AF's. The molecular weight of the factors produced by 3T3-SV40 cells was determined by chromatography on an agarose A-5 column, and ranged from 10^5 to more than 5×10^6 (*15*). These data suggested that the activity of the AF's resided in polydispersed polysaccharides or in polysaccharide-containing molecules. To test the effect of mucopolysaccharidases on the aggregation activity of supernatants, we incubated the supernatants for 24 hours at 38°C with different lots of bovine testicular hyalurondase (*16*) and titrated them on P388 cells. All of the aggregation activity produced by the cells, as is listed in Table 1, was completely destroyed or inactivated. This occurred when the concentrations of bovine hyaluronidase were as low as 0.1 to 1 USP unit per milliliter, an indication that the active component of the AF's may be hyaluronic acid (HA), chondrosulfates A or C, or a mixture of both. Initial experiments have shown that more than 90 percent of the aggregation activity released by mammalian fibroblastic cell lines is suppressed by streptococcal hyaluronidase, which is specific for HA (*17*).

Table 1. Quantitative assay of AF's released by various cells in culture P388 cells. The cells were routinely cultured in double-strength BME plus fetal calf serum (10 percent), in milk dilution bottles.

Cells	Cell type	Cell characteristics	Supernatant titers* (units of AF's/ml)
	Mouse		
P388	Lymphoblastic	Cell line	0
MEF	Fibroblastic	Secondary whole embryo culture	>200
NCTC 2472 (N1)	Fibroblastic		
NCTC 2555 (N2)	Epitheloid	Cell line	200
3T3	Fibroblastic	Cell line	200
3T3-SV40	Fibroblastic	Cell line	20,000
		SV40-transformed 3T3	30,000
	Rat		
REF	Fibroblastic	Secondary whole embryo culture	400
	Hamster		
HEF	Fibroblastic	Secondary whole embryo culture	400
Nil-2	Fibroblastic	Cell line	8,000
	Human		
WI-38-2RA	Epithelioid	SV40-transformed WI-38	1,200
HeLa	Epithelioid	Cell line, cervix carcinoma	200

* The P388 cells were collected after mechanical dissociation, washed three times in PBS, and suspended in BME. Cells (5×10^5) were distributed into 25-ml Erlenmeyer flasks containing 3 ml of twofold dilutions of supernatant in BME and were rotated at 60 rev/min on a gyratory shaker at 4°C for 24 hours. The P388 cells do not aggregate spontaneously in serum-free BME. The titer of the supernatants in units of AF's per milliliter is expressed as the reciprocal of the highest dilution at which P388 cells form aggregates of two or more cells. In undiluted supernatants or supernatants of subterminal dilutions, P388 cells formed large aggregates.

Several experiments were done to exclude the possibility that the suppression of aggregation by hyaluronidase was due to an effect on the P388 test cells rather than to inactivation of the AF's: (i) prior incubation of P388 cells with hyaluronidase did not decrease their ability to aggregate in the presence of active supernatants; (ii) the absence of proteolytic activity in the hyaluronidase preparations was shown by incubating ^{3}H-labeled proteins with the enzyme and measuring the release of trichloroacetic acid–soluble material; and (iii) after incubation with hyaluronidase but before assay on P388 cells, the supernatants were heated at 100°C for 20 minutes to inactivate the enzyme and possible traces of proteases.

In all experiments done with the AF's listed in Table 1, the cells used were either cell lines or secondary cultures of whole embryos. It was, therefore, important to determine whether the factors released from embryonic tissues in primary cultures were also MPS. For this reason, supernatants prepared from primary cultures of neuroretinal and liver cells of 10-day-old chicken embryos (*5*) were assayed on P388 cells and produced titers of 200 and 400 units of AF's per milliliter, respectively. These supernatants from the chick embryo cultures and those from 3T3-SV40 cells promoted the aggregation of 10-day-old chick embryo neuroretinal cells which had been

Table 2. Effects of aggregation factors (AF's) and hyaluronic acid (HA) on cell aggregation. Cell suspensions obtained either spontaneously (YACB), by mechanical dissociation (P388, N2, HC), or after a 2-minute treatment with $10^{-4}M$ ethylenediaminetetraacetic acid (N1), were washed three times in PBS by centrifugation at 100*g*. The degree of aggregation was tested in gyratory cultures at 4° or 37°C by suspending 10^6 cells in 3 ml of BME alone (spontaneous), or in undiluted supernatants from 3T3-SV40 cells with a titer of 30,000 units of AF per milliliter, or in 0.1 μg of HA per milliliter in 2 × BME (human umbilical cord, grade I, Sigma). Aggregation was rated from — (single cells) to ++++ (aggregates of thousands of cells).

Cells	Degree of aggregation		
	Spontaneous	AF	HA
P388	—	++++	++++
N2	±	++++	+++
N1	++	++++	++++
HC*	—	—	—†
YACB*	—	—	—†

* Mouse cell lines, histamine-releasing (HC) or lymphoblastic Moloney virus–transformed (YACB). † Hyaluronic acid at a final concentration of 1 mg/ml.

freshly treated with trypsin at 37°C (*18*). Dialyzed supernatants (*19*) also lacked specificity for tissue and species. Treatment with bovine hyaluronidase (1 USP unit per milliliter) suppressed the aggregation activity of all supernatants on both P388 and retinal cells —evidence that acid MPS are also responsible for the aggregation activity in chicken supernatants.

Because the activity of AF's contained in serums or in serum-free supernatants from cell cultures appeared to reside in acid MPS, we investigated the effects of HA on cell aggregation. Table 2 shows that HA has a strong aggregating effect, similar to that of other AF's, and that cells which are not aggregated by AF's are also not aggregated by HA. The amount of HA required to aggregate P388 cells was as low as 10 pg/ml; thus, it is improbable that the effect of HA is due to increased viscosity of the medium. In cells which have been treated with trypsin, no aggregation occurs at 4°C with either AF's or HA.

Many cell lines are known to release MPS (*20*) under conditions similar to those used in this study, and the AF's released by retinal cells are actually synthesized during culture (*21*). We have proposed that AF's are cell ligands and bind with receptors located at the cells' surfaces (*12*). Receptors for both AF's and HA are susceptible to proteases and thus may be similar. Some indication of the minimum length of the MPS molecule required for the aggregation effect is given by the observation that residues are ineffective after hyaluronidase digestion, whereas preparations of HA with a limiting viscosity of 190 cm^3/g (molecular weight of approximately 0.5 to 2×10^5) have a strong aggregation effect.

In our experiments, the AF's were apparently nonspecific in terms of species, cell type, or tissue. Nevertheless, it must be stressed that these data do not rule out either specificity of contact in cell adhesion, or the possibility that the extracellular AF's released by cells are composed of a mixture of specific and nonspecific factors.

The presence of MPS in the intercellular material or ground substance has been well established in vivo (*22*), and in fact many cells are covered with an MPS coat (*23*). The similarity between the AF's contained in serums and those released by cells in culture suggests that these factors may be involved in cell adhesion in vivo. The surface coats of some ascites and polyoma-transformed cells, of cells transformed by adenovirus 12 and by simian virus 40, and of spontaneously transformed cells (*24*) have been shown to be thicker than those of the control cells. It is assumed that this material consists partly of MPS. Moreover, chicken cells, after transformation by Rous sarcoma virus, release more MPS than previously (*25*). These observations agree with the fact that when malignant cells are tested for aggregation patterns, they give larger aggregates than control cells (*26*). Cell-to-cell binding by MPS may regulate in vitro phenomena, such as contact inhibition of movement and density-dependent inhibition of growth, or related in vivo phenomena in both normal and malignant cells.

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14. One milliliter of supernatant was mixed with an equal volume of receptor-destroying enzyme (Behringwerke Ag) or with 100 μg of one of the following enzymes: crystallized deoxyribonuclease I, ribonuclease A, twice-crystallized trypsin, crude collagenase (Worthington), or pronase, B grade (Calbiochem). After 4 hours of incubation at 37°C and *p*H 7.2, the preparations were heated at 100°C for 15 minutes before assay on P388 cells.
15. One milliliter of serum-free supernatant from 3T3-SV40 cells, containing 30,000 units of AF's/ml was applied to a 1.5 by 29 cm column of agarose A-5 equilibrated with PBS, *p*H 7.4. The column was eluted with this buffer and 3-ml fractions were collected and assayed for aggregation activity on P388 cells. About 80 percent of the AF's were found in the exclusion volume and in the next two fractions.
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27. B.P. is a fellow of the Damon Runyon Memorial Fund, Inc., at the Wistar Institute, on leave from the Institut d'Immunobiologie, Hôpital Broussais, Paris, France. V.D. is the recipient of faculty research award PRA-47 from the American Cancer Society. Supported in part by PHS research grant S01-RR-05540 and American Cancer Society grant E-89. We thank Dr. J. R. Whittaker of the Wistar Institute for his help in obtaining chick embryo neuroretinal cells, Dr. G. H. Warren, Wyeth Laboratories, for the streptococcal and bovine hyaluronidase, and Dr. E. Balazs for hyaluronic acid of different viscosities.

23 August 1971; revised 4 October 1971