percent when isovaleric acid was present in stimulus; 0 percent when absent). The differences between the percentages is significant at $P < .001$ in both cases.

The variations usually encountered in field experiments (Figs. 1 and 2) are in part due to known causes, such as diurnal, seasonal, and meteorological factors, even though the pronghorn were tested at the same time of day. Second, physiological short-term periodicities influence the readiness to scent marking: an animal will be less inclined to mark if it is ready to recline and chew its cud, whereas it will be more likely to mark after a period of rest. This has been demonstrated for black-tailed deer (*10*). Third, sudden noises of vehicles, airplanes, gunshots, and the like often cause the animals to assume the alert posture and change their response to odor stimuli. In view of these possible sources of variation, the results obtained are as consistent as they can be under the circumstances. No response value was discarded. We are aware of no other comparable study on chemical communication in any mammal under similar outdoor conditions.

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References and Notes

1. J. Schaffer, *Die Hautdrüsenorgane der Säugetiere* (Urban and Schwarzenberg, Berlin, 1940); R. F. Moy, *Am. J. Anat.* **129**, 65 (1970); *J. Mammal.* **52**, 441 (1971).
2. E. J. Prenzlow, D. L. Gilbert, F. A. Glover, *Colo. Dept. Game Fish Parks Spec. Rep. No. 17* (1968).
3. P. T. Bromley, *J. Mammal.* **50**, 81 (1969).
4. D. Müller-Schwarze and C. Müller-Schwarze, *Zool. Afr.* **1**, 257 (1972).
5. B. K. Gilbert, *Mammalia* **37**, 25 (1973).
6. D. W. Kitchen, thesis, University of Michigan (1972).
7. ——— and P. T. Bromley, in *The Behavior and Management of Ungulates*, V. Geist, Ed. (International Union for Conservation of Nature and Natural Resources, Copenhagen, in press).
8. D. Müller-Schwarze, C. Müller-Schwarze, W. L. Franklin, *Verh. Dtsch. Zool. Ges.* **66**, 146 (1973).
9. The average response value (ARV) for each tested stimulus was determined by adding points assigned to each of the four behavior patterns (sniffing, licking, marking, and thrashing) and dividing by N, the number of nylon rods. The points were based on a large number of observations wherein sniffing was 1 to 3 times more frequent than licking, 4 to 20 times more frequent than marking, and 5 to 30 times more frequent than thrashing. Points assigned, therefore, were 1 for sniffing, 1 to 3 for licking, 4 to 20 for marking, and 5 to 30 for thrashing. The exact number of points for each behavior was determined by the response to blanks that were presented with the samples of a particular test series. For example, in one particular series, in response to a total of 24 blanks, the animals sniffed 52 times, licked 27 times, marked 14 times, and thrashed 4 times; points given, therefore, were 1 for sniffing, 2 for licking, 4 for marking, and 13 for thrashing. In this series when isovaleric acid was presented at 0.1 μg on 6 rods, the animals sniffed 16 times, licked 9 times, marked 5 times, and thrashed 4 times. The total number of points, thus, was $(1 \times 16) + (2 \times 9) + (4 \times 5) + (13 \times 4)$, for a total of 106. The ARV for isovaleric acid was $106/6 = 17.7$ (see Fig. 1, left bar of compound **2**).
10. D. Müller-Schwarze, *Anim. Behav.* **19**, 141 (1971).
11. Supported by NSF grant GB 26640 to D.M.-S. The spectra were obtained by H. Jennison on a Hitachi RMU-6E mass spectrometer at 70 ev, which was obtained through an NSF grant.
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Brain Hyaluronidase: Changes in Activity during Chick Development

Abstract. *Development of embryonic chick brain is characterized by high levels of hyaluronidase activity and of hyaluronate, both of which decrease rapidly soon after the chick hatches. By analogy to other systems, it is proposed that the sequence of hyaluronate production and its enzymatic removal may have a developmental role in brain formation.*

Recent work in this laboratory has indicated that hyaluronate production and its subsequent enzymatic removal may be involved in the temporal control of cell migration and cell differentiation in a variety of embryonic and regenerating tissues. Studies of the regenerating newt limb (*1*) and of the developing chick cornea (*2*), limb bud, and axial skeleton (*3*) have led to the postulate that hyaluronate inhibits certain cell interactions necessary for tissue formation (*4*) and that enzymatic or hormonal influences, or both, release this inhibition at the appropriate time (*5, 6*). The possibility that hyaluronate turnover may be linked to the numerous waves of neuronal migration and differentiation during embryonic brain development is now being explored. We report here that relatively high levels of hyaluronidase activity and of hyaluronate are present during chick embryonic brain formation and that they diminish soon after hatching.

Previously, other investigators showed that mucopolysaccharides including hyaluronate are present in brain tissue of several species (*7*) and that they are major components of the extracellular microenvironment of brain cells (*8*). Levels of hyaluronate are known to vary considerably during development (*9*), and Margolis *et al.* (*10*) recently reported the presence of a hyaluronidase in the brains of newborn and adult rats and cows.

Hyaluronidase activities were determined by measurement of the terminal N-acetylhexosamine in oligosaccharides released during incubation of hyaluronate with homogenized chick brains from which endogenous substrate had been removed. Homogenization of chick brain in ten volumes of buffer (0.05M sodium acetate and 0.05M sodium chloride, pH 4.5) and centrifugation at 25,000g for 20 minutes resulted in the separation of brain hyaluronidase (localized in the precipitate) from brain endogenous substrate (localized in the supernatant). The precipitate containing the enzyme was resuspended with 0.1 percent Triton-X detergent in either 0.05M sodium acetate and 0.05M sodium chloride or 0.1M sodium formate and 0.15M sodium chloride buffers for 6 hours. All procedures were performed at 0°C. A portion (0.25 ml) of the Triton-X suspension (approximately 30 mg of protein per milliter) was pipetted into each tube with 200 μg of hyaluronate (Sigma Type IIIP) and incubated at 37°C for varying times up to 8 hours. Enzymatic activity was terminated by boiling at 100°C for 1 minute. Release of terminal reducing N-acetylhexosamine as a result of enzymatic action was measured according to Reissig *et al.* (*11*).

That brain hyaluronidase activities are higher in the embryo than in the hatched chick may be seen in a semiquantitative fashion in Fig. 1, which shows a determination of the enzymatic pH maximum in acetate and formate buffer systems. A comparison of these curves measuring the amount of terminal N-acetylhexosamine released after 8 hours of incubation shows that the embryo had approximately three to four times the enzymatic activity of the hatched chick when equal amounts and concentrations of protein were used for the range of pH values examined. Furthermore, a broad pH maximum, centered around pH 4.5, was ob-

tained with acetate buffer, and a sharper maximum at *p*H 3.7 was obtained with formate buffer. In the formate buffer system the enzyme showed twice the activity of that in the acetate buffer system. Enzymes from embryos and hatched chicks had identical maximums, which shows that these enzymes were probably qualitatively the same and that the change in hyaluronidase activity that occurred at hatching was probably a quantitative one.

For a more precise estimate of enzymatic activity during the stages of chick brain development, rate determinations were performed in both acetate (*p*H 4.5) and formate (*p*H 3.7) buffer systems. Figure 2 shows that hyaluronidase activities (measured with acetate buffer, *p*H 4.5) were constant from 7-day embryo to 19-day embryo (day prior to hatching), during which time the brain wet weight increased from 0.1 to 0.9 g. Two days after hatching, with an increase in this weight to only 0.95 g, the hyaluronidase activity had already decreased to 60 percent. At 8 days after the chicks hatched (brain, 1.20 g, wet weight) and 18 days after hatching (brain, 1.45 g, wet weight) there were further decreases in hyaluronidase activity to 30 and 25 percent, respectively, of the original value found in the prehatching period. Experiments in which formate buffer (*p*H 3.7) was used showed the same pattern of results, demonstrating again that 17-day embryos had four times the hyaluronidase activity that chicks had 8 days after hatching.

During this period of development of chick brain the protein content increased proportionately faster than did the wet weight of the brain, probably due to a decrease in hydration from embryo to young adult brain. In 7-day embryos protein was 2 percent of the wet weight, rising to a constant 5 percent for 15½ day-old, 19-day-old, and 22-day-old chicks (22 days is 2 days after hatching). Twenty-eight-day brain (8 days after the chicks hatched) contained 7 percent protein with 38-day ones (18 days after the chicks hatched) having 8 percent.

It follows, then, from the above results that the decrease in hyaluronidase activity is also apparent on a whole brain basis since (i) at the time of hatching (day 19 to 22), when a 40 percent decrease in activity per milligram of protein occurs, the increase in total protein per brain is only 5 percent; and (ii) from day 19 to day 28, over which time there is a fourfold

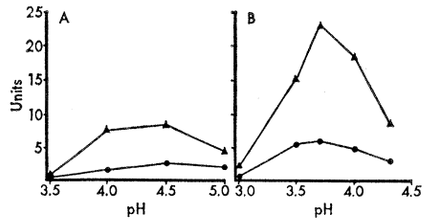

Fig. 1. Brain hyaluronidase: *p*H dependence of enzymatic activity comparing (A) acetate buffer (0.05*M* sodium acetate and 0.05*M* NaCl) and (B) formate buffer (0.1*M* sodium formate and 0.15*M* NaCl) for both 17-day embryos (▲) and 28-day-old chicks (8 days after hatching) (●). Incubations were for 8-hour periods with excess exogenous hyaluronate as described in the text. "Units" are defined as micrograms of *N*-acetylhexosamine released per milligram of protein of brain tissue.

decrease in activity per milligram of protein, the total protein per brain rises approximately twofold.

Brain tissue lysosomes contain the exoglycosidases, β-glucuronidase and β-*N*-acetylglucosaminidase, which by concerted action could give rise to further breakdown of the oligosaccharides produced by hyaluronidase, and thus augment the amount of reducing *N*-acetylhexosamine measurable in the assay used above. Consequently, control experiments were performed to rule out the possibility that the decrease in hyaluronidase activity from embryo to hatched chick might be due to high activities of these glycosidases in the embryo. Since exoglycosidase action would produce free *N*-acetylglucosamine from

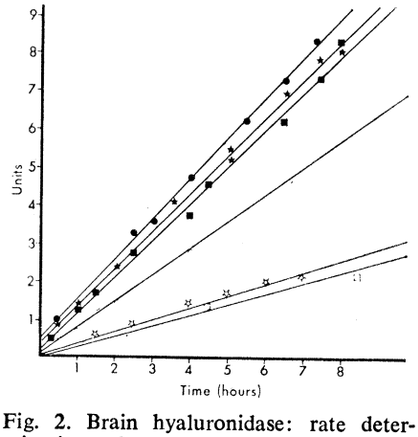

Fig. 2. Brain hyaluronidase: rate determinations of enzymatic activity (in acetate buffer, *p*H 4.5) for varying stages of chick development, comparing before-hatching stages to early after-hatching stages. These are 7½-day (●), 15-day (★), 19-day (■), 22-day (2 days after hatching) (○), 28-day (8 days after hatching) (☆), and 38-day (18 days after hatching) (□) brains. "Units" are defined as micrograms of *N*-acetylhexosamine released per milligram of protein of brain tissue.

the hyaluronate oligosaccharides, the breakdown products from incubation of 17-day embryo brain extracts with hyaluronate at *p*H 3.7 were examined by gel filtration on Sephadex G-25. At most, 20 percent of the reducing *N*-acetylhexosamine was obtained in the fractions corresponding to free *N*-acetylglucosamine, whereas the rest appeared in the tetrasaccharide or larger oligosaccharide fractions. Also, addition of 1.5 m*M* D-saccharolactone, which is a specific inhibitor of β-glucuronidase, did not significantly influence the hyaluronidase assay conducted in buffer systems having a *p*H of either 3.7 or 4.5. Under test conditions, this concentration of saccharolactone was shown to inhibit completely endogenous β-glucuronidase activity measured with either phenolphthalein glucuronidate or oligosaccharides, obtained by testicular hyaluronidase cleavage of hyaluronate, as substrate. These results are in accordance with those of Margolis *et al.* (*10*), who also showed that exoglycosidases did not significantly influence their assay for hyaluronidase in rat brain. The explanation may lie in the low effective substrate concentration of end groups in the intact and partially digested hyaluronate during the limited time of incubation.

Approximate hyaluronate amounts were determined by complete digestion of lipid-extracted, protein-free brain preparations with streptococcal hyaluronidase (which is specific for nonsulfated glycosaminoglycans) and measurement of the release of reducing *N*-acetylhexosamine. Lipid extraction and protein degradation were performed according to the method described by Margolis and Margolis (*12*) which included extraction with a mixture of chloroform and methanol (2 : 1), methanol and chloroform (2 : 1), ether drying, digestion with Pronase, and precipitation with 10 percent trichloroacetic acid (TCA). The supernatant from TCA precipitation was dialyzed for 24 hours against running distilled water and for 12 hours against a buffer composed of 0.1*M* sodium acetate and 0.1*M* sodium chloride (*p*H 5.0). Incubations to determine amounts of hyaluronate were performed at 37°C for 16 hours, with the use of 0.20 ml of the above preparation (obtained from 16 mg of lipid-free tissue, dry weight) and 0.5 mg of streptococcal hyaluronidase (gift of Dr. Paul Bell, Lederle Laboratories). Standards containing known amounts of hyaluronate were incubated under identical conditions.

This experiment showed that the decrease of hyaluronidase activity was mimicked by a decrease in hyaluronate concentration soon after the chicks hatched and that high levels of hyaluronidase activity exist in embryonic brain concurrently with high concentrations of hyaluronate. Seventeen-day embryos had a hyaluronate content of 6.9 μg per milligram of lipid-free tissue (dry weight) while chick brain 23 days old (3 days after the chicks hatched) and also brain from chicks 7 weeks after hatching contained 2.2 μg per milligram of lipid-free tissue (dry weight. The drop in hyaluronate may explain the decrease in hydration discussed in the previous section, since high hyaluronate concentrations are often associated with a high degree of hydration (*13*). Bondareff and Pysh (*14*) observed that the volume of extracellular space decreases during early development of the rat brain.

The parallel presence of both high concentrations of hyaluronate and high hyaluronidase activity during early development of brain presents a situation different from that encountered in the systems studied previously. In the regenerating newt limb (*1*) and the chick embryo cornea (*2*), limb, and vertebrae (*3*) the developmental sequences can be separated into a morphogenetic phase during which cells accumulate by proliferation and migration to a suitable location and a phase of overt differentiation [for example, see (*6*) and (*15*)]. The former phase is characterized by hyaluronate production, the latter by hyaluronidase activity. In the developing brain of the chick a series of migrations and differentiation continue throughout embryonic life (*16*). It is important to note that most, if not all, of the neuronal migrations have stopped in the chick at the time of hatching (*17*), while neuronal migration in mammalian species continue after birth (*18*). Thus in the chick coincident high concentrations of hyaluronate and high hyaluronidase activity correspond to an overlapping series of neuronal migrations and differentiations which give rise to the complex layers of brain cells. It is not yet known whether the enzyme and substrate are compartmentally separated in relation to cells that are differentiated as opposed to migrating or proliferating, or whether rapid turnover of hyaluronate is a uniform characteristic of the embryonic brain.

In our studies of the developmental sequences of skeletal and corneal development we have suggested that thyroxine may be involved in the control of hyaluronate concentrations and hyaluronidase activity (*5, 6*). This is of particular interest here since the effects of hypothyroidism in brain development are known to include a failure of the proper timing of neuronal migrations (external granule cells to internal granule cells) and a failure of the proper differentiation of other neurons (for example, Purkinje cells) (*19*).

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References and Notes

1. B. P. Toole and J. Gross, *Dev. Biol.* **25**, 57 (1971).
2. B. P. Toole and R. L. Trelstad, *ibid.* **26**, 28 (1971).
3. B. P. Toole, *ibid.* **29**, 321 (1972).
4. ———, G. Jackson, J. Gross, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1384 (1972).
5. B. P. Toole, *Science* **180**, 302 (1973).
6. ———, *Am. Zool.* **13**, 1061 (1973).
7. I. J. Young and L. G. Abood, *J. Neurochem.* **6**, 89 (1960); R. U. Margolis, *Biochim. Biophys. Acta* **141**, 91 (1967); M. Singh, E. V. Chandrasekaran, R. Cherian, B. K. Bachhawat, *J. Neurochem.* **16**, 1157 (1969).
8. F. O. Schmitt and F. E. Samson, *Neurosci. Res. Program Bull.* **7**, 277 (1969).
9. M. Singh and B. K. Bachhawat, *J. Neurochem.* **15**, 249 (1968); I. J. Young and J. T. Custod, *ibid.* **19**, 923 (1972).
10. R. U. Margolis, R. K. Margolis, R. Santella, D. M. Atherton, *ibid.* **19**, 2325 (1972).
11. J. L. Reissig, J. L. Strominger, L. F. LeLoir, *J. Biol. Chem.* **217**, 959 (1955).
12. R. K. Margolis and R. U. Margolis, *Biochemistry* **9**, 4389 (1970).
13. J. H. Fessler, *Biochem. J.* **76**, 124 (1960); J. A. Szirmai, in *The Amino Sugars*, R. W. Jeanloz and E. A. Balazs, Eds. (Academic Press, New York, 1966), vol. 2B, p. 129.
14. W. Bondareff and J. J. Pysh, *Anat. Rec.* **160**, 773 (1968).
15. E. Zwilling, *Dev. Biol.* **2** (Suppl.), 184 (1968).
16. J. H. LaVail and W. M. Cowan, *Brain Res.* **28**, 420 (1971).
17. J. Hanaway, *J. Comp. Neurol.* **131**, 1 (1967).
18. P. Rakic, *ibid.* **141**, 283 (1971).
19. J. Legrand, in *Hormones in Development*, M. Hamburgh and E. J. W. Barrington, Eds. (Appleton-Century-Crofts, New York, 1971), p. 381; M. Hamburgh, L. A. Mendoza, J. F. Burkart, F. Weil, in *ibid.*, p. 403.
20. Publication No. 607 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. Supported by the National Foundation-March of Dimes grant CRBS 229, and grant AM 3564 from the National Institute of Arthritis, Metabolism, and Digestive Diseases. J.R.P. is a trainee of the National Institute of Mental Health; B.P.T. is an Established Investigator of the American Heart Association.

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HL-A Antigens in Mummified Pre-Columbian Tissues

Abstract. *Tissue extracts of pre-Columbian mummies, from 500 to 2000 years old, were found to inhibit specific antibodies to HL-A. Two-thirds of the specimens tested gave positive results. Patterns of reactions obtained with different antiserums detecting the same antigen were concordant and consistent with known relations between HL-A antigens. The distribution of antigens found was similar to that observed in present-day descendants of the ancient populations studied. Although artifacts due to contaminating substances could have occurred, the reactions resembled in many respects those of HL-A antigens rather than those of nonspecific cross-reacting inhibitors. Development of a technique for HL-A typing of mummified remains may open new possibilities for anthropologic studies.*

Determination of the ABO blood groups has been used extensively in anthropology. It provides a way of identifying and characterizing populations on the basis of genes. The possibility of typing mummified remains for ABO blood groups was realized in 1937 (*1*). Since then, blood groups have been studied in mummified tissues from Egypt, the Aleutian Islands, the southwestern United States, and South America (*1, 2*).

The HL-A system of histocompatibility antigens is emerging as one of the most polymorphic genetic systems of man and has been recognized as a powerful tool for anthropologic studies (*3*). The HL-A system comprises two genetic loci, LA and Four, each having 15 or more alleles. New alleles are still being identified, especially in non-European populations.

In the study reported here HL-A antigens were determined in mummified tissues by using an antibody inhibition technique. Thirty-three specimes were used (Table 1). They were obtained through the courtesy of several anthropologists who documented their origins and cultural affiliations (*4*).

The tissues consisted of fragments of skin from various parts of the body or scalp. Extracts were prepared by shredding dry tissue in a VirTis homogenizer