

ern and ancient herbivorous reptiles, especially if, as seems likely, many large herbivorous dinosaurs also depended on microbial fermentation systems. In the absence of parental care, each new generation must actively contact a previous one for acquisition of fermentative microbes. Attraction of young animals to older conspecifics for this purpose could represent the mechanism for origin of multigenerational dinosaur social groups, whose fossilized footprints have recently come to light (22).

KATHERINE TROYER

Department of Zoology, University of California, Davis 95616, and
Smithsonian Tropical Research
Institute, APO Miami, Florida 34002

References and Notes

1. R. E. Hungate, *Annu. Rev. Ecol. Syst.* **6**, 39 (1975); R. H. McBee, *ibid.* **2**, 165 (1971).
2. R. E. Hungate, *The Rumen and Its Microbes* (Academic Press, New York, 1966).
3. A. S. Rand, in *The Ecology of Arboreal Folivores*, G. G. Montgomery, Ed. (Smithsonian Institution, Washington, D. C., 1978).
4. T. S. Parsons and J. E. Cameron, in *Biology of the Reptilia*, C. Gans and T. S. Parsons, Eds. (Academic Press, New York, 1977), vol. 6, p. 189; J. B. Iverson, *J. Morphol.* **163**, 79 (1980).
5. R. H. McBee, in *Iguanas of the World*, G. M. Burghardt and A. S. Rand, Eds. (Noyes, Park Ridge, N.J., in press).
6. A. S. Rand, *Copeia* **1968**, 552 (1968); G. G. Montgomery, A. S. Rand, M. E. Sunquist, *ibid.* **1973**, 620 (1973).
7. G. M. Burghardt, *Am. Zool.* **17**, 177 (1977).
8. H. W. Greene, A. S. Rand, *Science* **195**, 689 (1977); H. W. Greene, G. M. Burghardt, B. A. Dugan, A. S. Rand, *J. Herpetol.* **12**, 169 (1978).
9. Iguana eggs were excavated from nest sites near Barro Colorado Island, Panama, in late April or early May and kept at ambient temperature in the screened Kodak House animal room on Barro Colorado Island until they hatched. Hatchlings were weighed, measured, toe-clipped, and marked with a fiber-tip pen. Marked iguanas were caged according to their treatment groups; they were given free access to fresh *Tetragonia expansa* leaves; and hatchlings received a weekly vitamin D and calcium supplement. Cages were illuminated and heated by incandescent light bulbs on a cycle of 12 hours of light and 12 hours of darkness.
10. Weighed samples of the hindgut contents were preserved with 1 ml of 3 percent Formalin buffered to pH 7 and examined at a magnification of $\times 150$ for the presence of protozoa. Concentrations of microbes were determined from a 1:100 dilution of the same solution examined in a bacterial counting chamber at $\times 600$.
11. G. A. Schad, R. Knowles, E. Meerovitch, *Can. J. Microbiol.* **10**, 801 (1964).
12. R. E. Hungate, personal communication.
13. A. S. Rand, personal communication.
14. Samples were taken from the soil lining the nest chamber or from soil near but not contiguous to the nest. Mean concentrations of bacteria in the two sets of samples were: nest chamber soil ($N = 6$), 1.49×10^9 cells per gram; other soil ($N = 3$), 1.59×10^9 cells per gram; Mann-Whitney, $U = 7.5$; $P = .417$.
15. Outdoor cages were constructed by surrounding individual tree branches with nylon mesh fabric. They were located in a region of the lakeshore where adult iguanas were commonly seen feeding or sleeping. Food was provided as in (9).
16. Fisher's exact probability test, $P < .005$.
17. Hatchlings were caged individually and fed weighed amounts of *Tetragonia expansa* leaves daily. Feces were collected daily, dried, and weighed. Gross digestive efficiency [GDE = (food eaten - feces produced)/(food eaten) = (food retained)/(food consumed)] was estimated from dry weight of food eaten and feces produced daily.
18. For feces-fed hatchlings, GDE = 0.83 ($N = 4$); for controls, GDE = 0.79 ($N = 6$). Median test, $P = .024$.

19. Iguanas were sighted on their sleeping perches at night by means of a 200,000-candlepower hand-held light; they were observed from a boat passing along the lakeshore. Age, sex, perch height, and tree species were recorded, and location was plotted on an outline map for each iguana sighted. Distances between individuals were estimated for iguanas closer together than 10 m; greater distances were measured on the maps.
20. E. C. Pielou, *An Introduction to Mathematical Ecology* (Wiley, New York, 1969).
21. D. Werner (*Amblyrhynchus cristatus*) and J. B. Iverson (*Cyclura carinata*), personal communication.
22. R. T. Bakker, *Sci. Am.* **232**, 58 (April 1975);

Nature (London) **229**, 172 (1971); J. H. Ostrum, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **11**, 287 (1972); K. Troyer, in preparation.

23. I thank A. S. Rand for technical assistance and discussions. I thank G. M. Burghardt, A. S. Rand, M. J. Ryan, and J. A. Stamps for reading and commenting on the manuscript and the many temporary residents of Barro Colorado Island for discussions. Supported by a Regents fellowship from the University of California, Davis, by assistantships and other aid from the Smithsonian Tropical Research Institute, and by graduate research grants from the University of California, Davis, and from Sigma Xi.

30 November 1981

Autoantibodies to Insulin Receptor Spontaneously Develop as Anti-Idiotypes in Mice Immunized with Insulin

Abstract. Mice immunized with insulin developed antibodies to both insulin and the insulin receptor. The antibodies to insulin receptor displaced labeled insulin from insulin receptors and mimicked the actions of insulin in stimulating the oxidation of glucose and its incorporation into lipids, and in inhibiting lipolysis. The antibodies to insulin receptor could be blocked by or bound to the antibodies to insulin, and therefore were identified as anti-idiotypes. Thus, immunization against a hormone may activate spontaneously an idiotypic-anti-idiotypic network resulting in antibodies to the hormone receptor.

Several diseases of man can be related to the action of autoantibodies binding to receptors on the surface of normal cells of the individual. For example, patients with Graves' disease have antibodies that abnormally stimulate the thyroid gland by activating receptors for thyroid-stimulating hormone (TSH) (1). These antibodies to TSH receptor cause hypersecretion of thyroid hormones and hyperthyroidism. Myasthenia gravis is caused by antibodies that bind to and block the receptor for acetylcholine at the neuromuscular junction (2). Another instance of autoimmunity to receptor is exemplified by those patients with antibodies to the insulin receptor who suffer from severe insulin-resistant diabetes (3). The pathophysiology of autoimmunity to hormone receptors is not well understood. How might an individual's immune system be triggered to produce antibodies that bind to seemingly normal membrane components?

One explanation for receptor autoimmunity is that it arises in the course of anti-idiotypic regulation of the immune response. Animals may be immunized against the variable regions of specific antibody molecules (idiotypes) and so produce anti-idiotypes, antibodies directed against idiotypes (4). Some anti-idiotypes recognize the combining site of the idiotypic antibody (5). Thus, either antigen or anti-idiotypes may bind to the combining sites of idiotypic antibodies. Jerne (6) proposed that the immune system may be regulated by a network in which antigen induces production of idiotypes

which in turn induce anti-idiotypes that can feedback to shut off or modify the original idiotypic response. Antibodies to idiotypes can readily be prepared by immunizing animals against purified idiotypes; anti-idiotypes so produced have been shown to influence immune reactions (7). However, the spontaneous generation of anti-idiotypes in response to immunization against an antigen has seldom been detected (8), and therefore it has been difficult to demonstrate a physiological function for the hypothetical idiotypic-anti-idiotypic network.

Sege and Peterson (9) proposed that a hormone could be used as an antigen to stimulate idiotypic antibodies that recognize the hormone. Such antibodies might be used to induce anti-idiotypes that sterically fit the combining sites of the idiotypes. Thus, the anti-idiotypes could have a three-dimensional configuration similar to that of the hormone antigen, and such anti-idiotypes might be able to bind to the hormone receptor. Accordingly, Sege and Peterson immunized rabbits against rat antibodies to insulin and obtained anti-idiotypic antibodies that bound to rat insulin receptor.

We explored the possibility that animals immunized against insulin might spontaneously develop antibodies to their own insulin antibodies, and that some of these anti-idiotypes might interact with the insulin receptor. Our approach was to immunize mice against bovine or porcine insulins and to assay samples of their serum for the appearance of two factors: antibodies to insulin

and antibodies to the insulin receptor that mimic the action of insulin. Antibodies to insulin were detected in mice that were genetically able to respond to the particular immunizing insulin (10), and these antibodies increased to a relatively high titer after the mice were given a booster injection of insulin. However, in about 70 percent of the mice we also detected a remarkably high level of insulin-like activity by observing the effects of the serum on isolated rat fat cells in vitro. Figure 1A shows that lipogenesis, the incorporation of labeled glucose into lipid, was linearly related to the amount of test serum added to a suspension of fat cells and that 50 percent of maximum stimulation of lipogenesis was obtained at 1.1 μ l of test serum. Because insulin produced 50 percent of maximum stimulation at a concentration of 0.3 ng/ml in this assay (not shown), it can be calculated that the test serum contained insulin-like activity equivalent to 270 ng of insulin per milliliter. The test serum competed with insulin for insulin receptors and displaced labeled insulin from the fat cells (Fig. 1B). Control serum obtained from mice not immunized against insulin showed neither of these effects (Fig. 1, A and B). Mice genetically incapable of mounting an immune response to porcine or bovine insulin did not develop insulinlike activity in response to injection of these insulins; only the serum of mice that made antibodies to insulin developed insulinlike activity.

We found that the insulinlike activity was a property of immunoglobulin G

(IgG) molecules. Serum samples were passed through chromatography columns of protein A bound to Sepharose. Protein A binds specifically to the Fc portion of antibodies, primarily of the IgG class (11). All of the insulinlike activity reflected by displacement of insulin (Fig. 1B), glucose oxidation (12), lipogenesis (Table 1 and Fig. 1A), and inhibition of lipolysis bound to and could be eluted from the protein A-Sepharose column (Table 1). The eluted material was identified as IgG by means of polyacrylamide gel electrophoresis (13). No insulinlike activity was detectable in the effluent that was not bound.

The IgG with insulinlike activity logically could be attributed to two different kinds of antibodies. Antibodies to insulin (idiotypes) complexed with insulin could be envisioned as binding to the insulin receptor because of the insulin component of the complex. In this case the antibody would recognize only the hormone, whereas the insulin would interact with its receptor on the fat cell. Alternatively, antibodies to idiotypes with the three-dimensional configuration of insulin might bind to the insulin receptor directly and thus function as antibodies to the receptor without the mediation of insulin. In experiments designed to discriminate between these possibilities we found that insulin and the antibodies with insulinlike activity seemed to interact with different sites on the insulin receptor. Mild trypsinization of fat cells, a treatment that greatly decreases the ability of the cells to respond to insulin

(14), did not affect the ability of the fat cells to respond to the insulinlike antibody (Fig. 1C). The insulinlike effects of the lectin concanavalin A are also unaffected by treating target fat cells with low concentrations of trypsin (15). Hence, it is conceivable that the insulinlike antibodies interact with the same trypsin-resistant site on the receptor as does concanavalin A. In any case, these results argue against the possibility that insulin itself complexed to antibodies to insulin could have mediated the interaction between the insulin receptor and the antibodies with insulinlike activity. We directly analyzed this point by dissociating any immune complexes that might exist between insulin and antibodies to insulin and separating the dissociated insulin by gel chromatography (Table 1). We found that 85 percent of the insulinlike lipogenic activity was associated with the high molecular weight material that was free of insulin. Hence, there was no evidence that the insulinlike activity was related to immune complexes.

Evidence that the insulinlike antibodies to insulin receptor were anti-idiotypes was derived from the finding that lipogenesis was inhibited by adding affinity-purified mouse antibodies to insulin to the serum (Table 1). We also separated physically the insulinlike antibodies from the insulin antibodies. When we passed the two types of antibodies through a column of agarose covalently bound to either normal guinea pig serum or guinea pig antiserum to insulin, the mouse insulin antibodies were not re-

Table 1. Insulin antibody and insulinlike lipogenic activities of serum from test mice immunized against insulin. The titer of the insulin antibodies was determined as described (27). Adipocytes were prepared and lipogenesis was measured as described in the legend to Fig. 1.

Treatment of test serum	Serum fraction	Insulin antibody titer (dilution ⁻¹)	Lipogenesis (micro-liters per 50 percent activity)	Activity of serum fraction relative to activity of whole serum (%)	
				Insulin antibody	Lipogenesis
None	Whole	10 ⁴	1.1	100	100
Dissociation of immune complexes*	Void volume	N.D.	1.3	N.D.	85
Addition of mouse antibody to insulin (12 μ g/ml) [†]	Whole	N.D.	2.2	N.D.	50
	Effluent	N.D.	>10	N.D.	0
	Eluate	N.D.	1.1	N.D.	100
Insulin antiserum column [‡]	Effluent	10 ⁴	1.6	100	70
	Eluate	<1.0	3.7	0	30
	Effluent	<1.0	1.3	0	85
Insulin column [‡]	Eluate	10 ⁴	>10	100	0

*Test serum (50 μ l) was acidified to pH 2.5. Some [¹²⁵I]insulin (40,000 count/min) was loaded on a Sephadex G-100 column (20 by 0.5 cm) which was preequilibrated and developed with 0.1M acetic acid and 0.2 percent bovine serum albumin (BSA) (pH 2.5). The void volume fractions prior to these that contained radioactive insulin were pooled, neutralized, and examined for bioactivity. [†]Mouse IgG antibody to insulin was adsorbed on an agarose-insulin column, eluted with 0.1M acetic acid and neutralized with solid NaHCO₃. This fraction blocked insulin-mediated lipogenesis at a molar ratio of IgG to insulin of 2000 to 1 (22). [‡]Test serum (30 μ l) was loaded on a protein A-Sepharose column (Pharmacia; 1.0 by 0.5 cm) that was preequilibrated and washed with a buffer consisting of Krebs-Ringer bicarbonate and 2 percent BSA (pH 7.4). A fraction of 2.0 ml was collected (effluent). The column was then washed with 0.1M acetic acid, and a 2.5-ml fraction (eluate) was collected and immediately neutralized with solid NaHCO₃ to pH 7.4. Essentially the same procedure was applied to the insulin-agarose affinity column (insulin-succinyl-diaminodipropylamine-agarose), prepared as described (23) and to the insulin antiserum-agarose column (prepared from guinea pig antiserum to pork insulin (Miles-Yeda) covalently linked to Sepharose-polyacrylamide hydrazide (24)). Control columns of Sepharose or agarose alone or nonrelevant serum covalently linked to agarose did not adsorb any of the activities identified in this table. Volumes of the fractions that emerged from the columns were measured, and the results computed as the percentage activity relative to that obtained from the test serum before column separation. The results are representative of experiments done with seven individual samples of test serum.

tarded at all, whereas 30 percent of the insulinlike activity was bound. This bound activity was quantitatively eluted from the column and the eluate contained essentially none of the antibody to insulin. Using another affinity column, made of insulin-agarose (Table 1), we found that only the antibodies to insulin bound to and could be eluted from this column.

These results indicate that anti-idiotypes can be generated spontaneously in response to immunization against antigen and lend support to the network hypothesis of Jerne (6). We suspect that our success in detecting spontaneous idiotypes can be attributed to the fact that we measured their insulinlike effects on the metabolism of fat cells. The immunological assays of anti-idiotypic antibodies now available probably lack the degree of amplification inherent in the metabolic assays that we used. It is not

certain that anti-idiotypes function to regulate immune responses as proposed by Jerne (6), nor is it clear how idiotypes and anti-idiotypes can coexist and function in the same serum without neutralizing each other. However, this study does show that anti-idiotypes can participate in a chain of autoimmune reactions leading to autoimmunity to receptor. Murine, human, and ungulate insulins differ by very few amino acid substitutions (16) that are concentrated at the pole of the molecule, and this region apparently does not bind to the hormone receptor (17). Antibodies produced in response to one insulin cross-react fully with the other insulins (10, 18), including self-insulin. Hence, some if not most antibodies to insulin can recognize antigenic determinants on self-insulin and, thus, are autoantibodies. T lymphocytes can also recognize self-insulin and express autoimmunity to insulin (19). Spontane-

ous anti-idiotypes recognizing self-antibodies constitute a second element of autoimmunity. A third example of autoimmunity in the network is the interaction of these anti-idiotypes with the hormone receptor. A similar network of autoimmunity could account for spontaneous antibodies to hormone receptors in humans. Hence, some patients apparently suffering from autoimmunity to TSH or to insulin receptors may be expressing a primary autoimmune response against the hormones. Mice demonstrating very high levels of insulinlike activity appeared to be clinically healthy provided that they were allowed free access to food. Therefore, antibodies to insulin receptor may be clinically inapparent, in some humans as well as in mice. Humans treated with exogenous insulin often develop antibodies to insulin (20). Might not some of these persons also develop anti-idiotypes with antireceptor activity? How do men or mice adapt to such receptor antibodies so that these molecules remain clinically covert? What effects might such antibodies have on the clinical course of diabetes?

YORAM SHECHTER

Department of Hormone Research,
Weizmann Institute of Science,
Rehovot 76100, Israel

RUTH MARON

Department of Cell Biology,
Weizmann Institute of Science

DANA ELIAS

Department of Hormone Research,
Weizmann Institute of Science

IRUN R. COHEN

Department of Cell Biology,
Weizmann Institute of Science

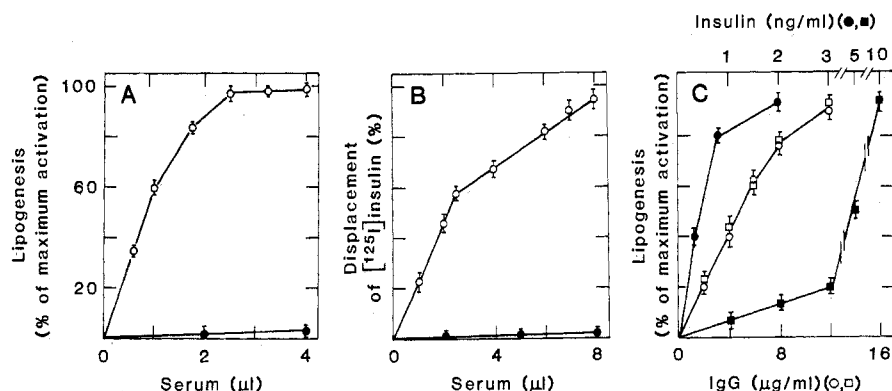


Fig. 1. Lipogenesis and displacement of labeled insulin from fat cells by mouse test serum and its IgG fraction. Mice (10 to 12 weeks of age) of strains BALB/c (H-2^b) or (BALB/c × C57BL/6)F₁ (H-2^d × H-2^b) were immunized with bovine or porcine insulin by injection in the hind foot pads with 25 μg of insulin emulsified in complete Freund's adjuvant (10). Three weeks later the mice received an intraperitoneal booster dose of the same insulin in adjuvant, and blood was obtained 10 days later. Control mice were immunized with complete Freund's adjuvant without insulin. Test (○) or control (●) serum samples were assayed for antibodies to insulin as described in Table 1 and for (A) lipogenesis or (B) displacement of [¹²⁵I]insulin. (A) From 120-g male Wistar rats, suspensions of adipocytes were prepared containing 0.5 × 10⁴ cells per milliliter in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.3 percent bovine serum albumin, as described (25). The adipocytes were incubated in plastic vials (5 ml per vial) for 2 hours at 37°C in 95 percent O₂ and 5 percent CO₂, with 0.2 mM [U-¹⁴C]glucose and increasing concentrations of serum from test or control mice. The reaction was terminated by adding toluene-based scintillation fluid directly into the incubation vials and counting the extracted lipids (26). Maximum lipogenesis, obtained by incubation with 10 ng of insulin per milliliter, was 480 percent of control values without added hormone. Lipogenesis produced by added serum was computed as the percentage of activation relative to the maximum produced by 10 ng of insulin per milliliter. (B) Suspensions of adipocytes (3 × 10⁵ cells per milliliter) were incubated with [¹²⁵I]insulin (0.3 ng/ml, 200,000 count/min-ng) and test or control serum samples in the above buffer for 1 hour at room temperature in a volume of 0.5 ml. The adipocytes were then separated from unbound hormone on Millipore (EGWP, 0.2 μm) filters, washed with ice-cold buffer, and counted for their radioactive content as described (21). The extent of binding was 1.7 fmole per 3 × 10⁵ cells of which 76 percent was specific (displaced by 1 μM native insulin). Results are expressed as percentage of displacement of specifically bound labeled insulin. Serum that had a relatively high titer of insulin bodies (10⁻³ dilution) but no insulinlike activity did not displace labeled insulin from fat cells (not shown). (C) Lipogenesis by IgG (○, □) and insulin (●, ■) in control (○, ●) and trypsinized (□, ■) fat cells. Immunoglobulin G from the test serum was isolated by adsorption on a Sepharose-protein A affinity column, as described in Table 1. The adsorbed IgG fraction was eluted with 0.1M acetic acid and neutralized with NaHCO₃. Trypsinization was carried out by exposing the fat cell suspension to trypsin (30 μg/ml) for 10 minutes at 37°C; ovomucoid (80 μg/ml) was then added (14). The results of (A), (B), and (C) are the means (± standard deviation) of triplicate samples of individual test and control sera. Similar results were obtained for 18 of 24 serum samples tested.

References and Notes

1. M. T. W. Hearn, *Trends Biochem. Sci.* **5**, 75 (1980).
2. S. Fuchs, *Curr. Top. Microbiol. Immunol.* **85**, 1 (1979).
3. J. S. Flier, C. R. Kahn, J. Roth, R. S. Bar, *J. Clin. Invest.* **58**, 1442 (1976).
4. Y. Oudin and M. Michael, *J. Exp. Med.* **130**, 595 (1969); H. G. Kunkel, M. Mannik, R. C. Williams, *Science* **140**, 1218 (1963).
5. B. W. Brient, J. Haimovich, A. Nisonoff, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3136 (1971).
6. N. K. Jerne, *Ann. Immunol. Inst. Pasteur* **125C**, 373 (1974).
7. K. Eichmann, *Adv. Immunol.* **26**, 195 (1978); H. Binz and H. Wigzell, *J. Exp. Med.* **144**, 1438 (1976).
8. L. Klusken and H. Kohler, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 5083 (1974); A. F. Schräter, E. A. Gold, G. J. Thorbecke, G. W. Siskind, *J. Exp. Med.* **150**, 138 (1979); J. S. Cowdery and A. D. Steinberg, *J. Immunol.* **126**, 2136 (1981).
9. K. Sege and P. A. Peterson, *Nature (London)* **271**, 167 (1978); *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2443 (1978).
10. K. Keck, *Nature (London)* **254**, 78 (1975); *Eur. J. Immunol.* **7**, 811 (1977); I. R. Cohen and J. Talmon, *ibid.* **10**, 284 (1980).
11. S. W. Kessler, *J. Immunol.* **115**, 1617 (1975).
12. The test serum and its IgG fraction (Table 1) also stimulated glucose oxidation and inhibited lipolysis. Glucose oxidation was carried out with suspensions of adipocytes (3 × 10⁵ cells per milliliter) for 2 hours at 37°C with 0.2 mM [U-¹⁴C]glucose. The amount of glucose converted to CO₂ was determined as described (25). Maximum glucose oxidation in the presence of insulin

- was 3.5-fold higher than in the absence of the hormone. Assay of lipolysis was carried out for 3 hours in the absence of the hormone, and for 3 hours at 37°C in 5-ml vials containing 3×10^5 cells, $0.4 \mu\text{M}$ isoproterenol, and increasing concentrations of the test serum. Portions of the medium were then taken, bovine serum albumin was removed by trichloroacetic acid precipitation, and the glycerol content was determined by the triglyceride C-37 Rapid-Stat test (see *Pierce Catalog*, 1976). The amount of glycerol released was 12 and 140 nmole per 3×10^5 cells per 3 hours in the absence and the presence of $0.4 \mu\text{M}$ isoproterenol, respectively. Insulin at 10 ng/ml inhibited 87 percent of the glycerol released. The titer of antibodies to insulin was determined by a solid phase radioimmunoassay (27). Half-maximum activity was obtained at 1.0 μl and 1.5 μl of the test serum per milliliter for glucose oxidation and inhibition of lipolysis, respectively.
13. The fraction eluted from the protein A-Sepharose column was pure IgG as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and mercaptoethanol performed as described by U. K. Laemmli [*Nature (London)* **227**, 680 (1970)]. Only the heavy and light chains of IgG were detectable on the gel. No bands that corresponded to insulin were seen.
 14. T. Kono and F. W. Barham, *J. Biol. Chem.* **246**, 6210 (1971); P. Cuatrecasas, *ibid.*, p. 6522.
 15. P. Cuatrecasas, *ibid.* **248**, 3528 (1973).
 16. M. O. Dayhoff, Ed., *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Washington, D.C., 1972), vol. 5.
 17. T. L. Blundell, G. G. Dodson, D. C. Hodgkin, D. A. Mervulo, *Adv. Protein Chem.* **26**, 279 (1972); G. Ranghino, J. Talmon, A. Yonath, I. R. Cohen, in *Structural Aspects of Recognition and Assembly in Biological Macromolecules*, M. Balaban, Ed. (Balaban, Jerusalem, 1981), p. 263; P. De Meyts, E. Van Obberghen *et al.*, *Nature (London)* **273**, 504 (1978).
 18. M. A. Barcinski and A. S. Rosenthal, *J. Exp. Med.* **145**, 726 (1977).
 19. I. R. Cohen, J. Talmon, V. Lev-Ram, A. Ben-Nun, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4066 (1979).
 20. S. A. Berson and R. S. Yallow, *Diabetes* **6**, 402 (1957).
 21. P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1264 (1971).
 22. Y. Shechter, in preparation.
 23. P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1277 (1972).
 24. T. Miron, W. G. Carter, M. Wilchek, *J. Solid-Phase Biochem.* **1**, 225 (1976).
 25. M. Rodbell, *J. Biol. Chem.* **239**, 375 (1964).
 26. A. J. Moody, M. A. Stan, M. Stan, J. Gliemann, *Horm. Metab. Res.* **6**, 12 (1974).
 27. Z. Eshhar, G. Strossman, T. Waks, E. Mozes, *Cell. Immunol.* **47**, 378 (1979).
 28. This research was supported by NIH grant AM 26766. We thank M. Margalit and P. Reitman for technical assistance, and R. Lifshitz and M. Schwartz for helpful discussions.

9 November 1981; revised 25 January 1982

Calcium Ionophore Polarizes Ooplasmic Segregation in Ascidian Eggs

Abstract. *Calcium ionophore A23187 promotes ooplasmic segregation and orange crescent formation in eggs of the ascidian *Boltenia villosa*. When eggs were exposed to a gradient of A23187 the orange crescent was induced to form in the region corresponding to the highest concentration of ionophore. This result is consistent with the hypothesis that a local increase in intracellular calcium polarizes cytoplasmic localization in the ascidian embryo.*

Cell fate during early embryogenesis is thought to be determined by the localization and segregation of cytoplasmic components (1). The distribution of these components in many embryos is established by an extensive episode of cytoplasmic rearrangement, known as ooplasmic segregation, which is initiated shortly after fertilization. Although ooplasmic segregation is often one of the earliest visible indications of the future embryonic axis, little is known about how the various cytoplasmic components are directed to specific regions of the egg. It was recently proposed that ooplasmic segregation may be organized by an intracellular gradient of free calcium (2). This proposal is supported by experiments with developing fucoid eggs which suggest that rhizoids are extended toward areas of high calcium concentration (3, 4). Transient elevations in intracellular calcium may also play an important role in the activation and early development of animal eggs (5). The present study provides evidence that the segregation of cytoplasmic regions with specific morphogenetic fates and the subsequent expression of bilateral symmetry in ascidian eggs are directed by a

local increase in intracellular calcium at the time of fertilization.

We investigated the role of calcium in ooplasmic segregation by treating *Boltenia villosa* eggs with the divalent ionophore A23187 (6). *Boltenia villosa* eggs were selected because, after fertilization, they exhibit a pronounced segrega-

tion of pigmented cytoplasmic regions which is readily followed by light microscopy. Ooplasmic segregation is initiated by the streaming of cortical cytoplasm, composed of orange pigment granules and associated mitochondria, into the vegetal hemisphere of the egg, where it collects around the point of sperm entry as an orange crescent. A clear cytoplasm, derived from the germinal vesicle, flows into the vegetal hemisphere in the wake of the orange cytoplasm. The clear cytoplasm temporarily forms another crescent immediately inside the orange crescent and eventually returns to the animal hemisphere with the male pronucleus. A cytoplasmic lobe is also protruded from the vegetal region of the egg at the time of maximal crescent development. The plane of the first cleavage furrow bisects the orange crescent, dividing the zygote into right and left halves characteristic of the bilaterally symmetric ascidian embryo. The developmental fate of the orange crescent cytoplasm of *B. villosa* eggs is identical to that of the yellow crescent cytoplasm of *Styela partita* eggs, the subject of Conklin's classical observations on ooplasmic segregation (7). Orange crescent cytoplasm is specifically partitioned to the precursors of the tail muscle and mesenchyme cell lineages in tadpole larvae.

Ionophore A23187 was previously shown to activate ascidian eggs and promote several partial cleavages (8) but, since the species investigated lacked eggs with pigmented cytoplasmic regions, no information on the ooplasmic segregation was obtained. In order to obtain this information, eggs were dissected from the gonads, washed with artificial seawater (ASW), and placed in petri dishes containing A23187 dissolved in ASW at several different concentrations (9). When the eggs were examined 20 to 30 minutes later a large proportion had developed orange crescents. The highest proportion of eggs with orange crescents (65 to 80 percent) was obtained with $4 \mu\text{M}$ A23187. Less than 3 percent of eggs incubated in ASW lacking A23187 developed orange crescents. Orange crescent formation did not depend on the concentration of external calcium, sodium, or hydrogen ions since it was not affected when eggs were treated with A23187 in calcium-free ASW containing 3 mM EGTA, in ASW in which choline was substituted for sodium, or in

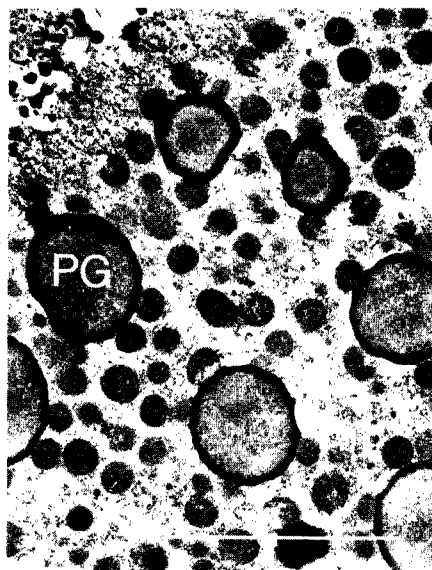


Fig. 1. Electron micrograph of the orange crescent region of an A23187-activated egg. Pigment granules (PG) with associated mitochondria are present. Scale bar, 10 μm .