a variety of cell types (*15*), but not as a component of an epithelium engaged solely in electrolyte secretion. The abundant mucopolysaccharide bordering the salt-secreting cells in the lachrymal gland of the turtle suggests strongly that it is linked with electrolyte secretion. Staining reactions indicative of mucopolysaccharide have also been reported in the secretory cells of the salt gland of the duck (*7*) and at the borders of the secretory cells in the rectal gland of the dogfish (*16*).

Intercellular channels bearing some resemblance to those described in this report have been observed in the ciliary epithelium of the eye (*17*) and between the clear cells of eccrine sweat glands (*18*). The tubule cells of the rectal gland of elasmobranchs (*18*, *19*) as well as the cells lining the central ducts or canals in the salt gland of the herring gull bear interdigitating processes along their margins (*20*). All of these cells are involved in the secretion of electrolytes. In these epithelia, however, the intercellular channels are less elaborate than those in the salt glands of marine turtles; and mucopolysaccharide has been reported only between the secretory cells in the rectal gland of the dogfish (*18*). Thus the abundance, the complexity, and the mucopolysaccharide content of the intercellular channels in the salt gland of the turtle seem to make them unique.

In the salt glands of marine birds the principal secretory cells have deep clefts along their basal and lateral surfaces (*6*). Adjacent cells bear clefts that complement each other, and this results in an extensive intermeshing of the cell surfaces. These interfolded cell processes containing both mitochondria and agranular endoplasmic reticulum serve to increase greatly the absorptive surface of the secretory cell (*6*); and they are analogous to the microvilli which fringe the salt-secreting cells in turtles. Thus, the architecture of the salt-secreting cells of marine birds and reptiles seem to represent two independent solutions to the problem of electrolyte excretion. The cells lining the convoluted tubules of the kidney (*21*) resemble the salt-secreting cells of birds, while the secretory cells of the rectal glands of elasmobranchs (*16*, *19*) resemble those of reptiles. The major differences in the fine structure of these cells are restricted primarily to the cell surface, while their contents are essentially similar.

The bulk of the evidence available from studies of fine structure shows that abundant mitochondria and an intracellular system of agranular membranes are the hallmark of cells that from physiological investigations are known to concentrate electrolytes. These organelles are both conspicuous and abundant in the secretory cells of the salt glands of marine birds (*6*), the rectal glands of elasmobranchs (*16*, *19*), the chloride cells of fishes (*22*), the salt cells of crustacean gills (*23*), the cells of the anal papillae of mosquito larvae (*24*), and the cells of the convoluted tubules of the kidney (*21*). The secretory cells of the salt glands of marine turtles are no exception to this rule and offer further evidence that these organelles participate directly in the secretion of electrolytes.

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Geotropism: Its Orienting Force

Abstract. *Seeds of* Pisum sativum *L. and other species were germinated and grown for five or more days on a continuously rotating vertical-axis turntable that developed a maximum centrifugal force of 1.79*g. *Shoot (epicotyl) orientation in darkness was parallel to the resultant gravitational field. This is presented as confirmation of the hypothesis that the orienting force of geotropism of the higher plants is the inertial force of gravity.*

When a plant organ grows vertically (or at some particular angle to the vertical) this oriented growth phenomenon is referred to as geotropism. The purpose of this study is to establish on a firm footing that the normal geotropic response of the higher plants is a response to the inertial force of gravity and not to some other stimulus —known or unknown—parallel to it. It has been demonstrated repeatedly, and perhaps most elegantly by Fitting (*1*), that the geotropic response is to a vertical force. That this force is gravity has been accepted almost intuitively, but surprisingly, with almost no experimental verification.

The hypothesis that gravity is the orienting force for geotropism is perhaps best tested experimentally by growing a plant in the reoriented gravitational field attained by the use of a centrifuge. Should the geotropic orienting force be other (or partially other) than gravity, then the orientation of the plant would, of course, not be parallel to the resultant gravitational force (*2*). Such experiments have been reported apparently only seven times (*3–9*), with curious results. Two of the seven investigators (*4*, *6*) became convinced that they could reject the hypothesis, while the remaining five felt that it could be accepted. A careful analysis of the seven sets of data indicates that confirmation of the hypothesis was not justified by the experimental results in at least four of the cases (*3*, *4*, *6*, *9*), nor perhaps in some of the others. The reports in three cases (*3*, *4*, *9*) indicate that phototropic responses exerted an unrecognized influence on the orientation of the plants; in two cases (*6*, *8*) the centrifugal forces actually involved seem to have been reported incorrectly; in one case (*4*) the data were miscalculated and misinterpreted. There

was a paucity of observations in two cases (*7, 9*) and excessive variability of individual observations in three cases (*4–6*).

Other approaches to the question of whether gravity is the orienting force for geotropism have been attempted from time to time (*10*), but can be objected to on theoretical grounds.

As a result of the differences in the literature, I decided to obtain further data to test the hypothesis in question. To this end I employed a vertical-axis centrifuge (*11*) set up within a greenhouse. The turntable rotated at 0.564 rev/sec and had an effective radius of 140 cm. Centrifugal forces were varied to a maximum of 1.79*g* by attaching plants at various distances from the center. Centrifuging was continuous in all experiments. Constant conditions of moisture were obtained by wick action from reservoirs. Darkness was maintained throughout by the use of light-proof metal containers (having a water-saturated internal atmosphere). In a subsidiary series, the plants were exposed to natural illumination augmented from 8 a.m. to midnight by banks of incandescent lights suspended over the turntable. Wind was eliminated in the dark-grown series by the metal containers and in the light-grown series by methyl methacrylate ("Plexiglas") cylinders. Temperature was maintained at 21° ± 3°C.

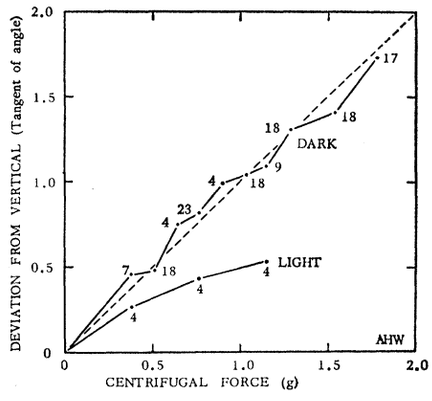

Fig. 1. Deviation from vertical of shoots (epicotyls) of *Pisum sativum* L. germinated and grown on a vertical-axis centrifuge (under windless conditions). The series grown in the dark was centrifuged continuously for 5½ days; the series grown in the light for 8 days, the seedlings being illuminated from above. The numbers signify the number of observations upon which each plotted point is based. The broken line is the deviation from the vertical of the resultant gravitational field.

Observations were made on the shoot orientation of seedlings germinated and grown directly on the rotating centrifuge from 5 to 20 days. The results reported are for *Pisum sativum* L. cv. Alaska. Completely comparable results were obtained with diverse other plants including *Avena sativa* L. and *Pinus strobus* L. The pea seeds were first soaked for 24 hours and then individually skewered with brass pins through their cotyledons to filter paper stretched over cork blocks. The specimens grown in the dark were then centrifuged for 5½ days, and a smaller series of specimens was grown in the light and centrifuged for 8 days. A specially constructed protractor was then used to determine the orientation of the shoots (epicotyls), measured in degrees of deviation from the vertical.

The data are best examined when the observed angular deviations from the vertical are plotted as their tangent transformations against the centrifugal force applied. Thus transformed, the angles of deviation should be directly and linearly proportional to the centrifugal force applied if the hypothesis is valid and orientation is parallel to the resultant gravitational field.

During 5½ days in total darkness, the shoots grew parallel to the resultant gravitational force (Fig. 1, dark). When the centrifugation occurred with overhead illumination, however, the plants also responded phototropically. The interaction of the upward phototropic response with the response to the resultant gravitational force reduced the deviation of centrifuged epicotyls from the vertical orientation (Fig. 1, light). Thus it can be seen that the pea shoots did indeed grow parallel to the resultant gravitational force as long as the confounding influence of a unilateral light source was excluded. A straight-line curve passing through the origin was fitted algebraically to the transformed data by the method of least squares. The calculated equation for the plants grown in the dark was: $D_T = 0.970\ C$ (where D_T is the tangent of the observed angle of deviation and C is the magnitude of the applied centrifugal force in units of *g*). Statistical analysis (*12*) indicated the validity of summarizing the data by a straight line arising from the origin and having a slope of 1.

To sum up, these data help to confirm experimentally the logical but almost intuitive support given by many to the hypothesis that the orienting force for the geotropism of plants is an inertial one. Actual past investigations into the subject have been few and contradictory, perhaps because of unrecognized and uncontrolled phototropic effects and failure to determine the centrifugal forces actually acting upon the plants.

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11. The centrifuge consisted of a 1.9-cm plywood turntable attached to the propeller shaft and bearing of an Allison airplane driven by a worm gear and wheel arrangement attached to a single-phase, 115-volt, 60-cycle, 1725-rev/min, ¼-horsepower General Electric motor equipped with a 50:1 American motorized worm-gear speed reducer.
12. The data from the dark-grown series were subjected to analysis of variance, significance being considered achieved at the 1

percent level. For purposes of analysis a second line was fitted to the data without regard to origin: $D_T = 0.106 + 0.886\ C$. Parallel analyses were performed with this curve and the one forced through the origin. Differences among levels of treatment (applied centrifugal force) were found to be significant for both lines. When the linear component of the treatment term was tested separately, it was found to be significant for either line, while the remaining higher order components (or nonlinear variation) were found to be nonsignificant and could be considered as residual scatter. The slope of either line was nonsignificantly different from a value of 1. The intercept value was nonsignificantly different from 0. It must be mentioned that the variances were found to be moderately heteroscedastic, increasing somewhat with increasing magnitude of applied centrifugal force; the "maximum-*F*-ratio" test [H. A. David, *Biometrika* **39**, 422 (1952)] indicated significance at the 5 percent level. This condition somewhat reduces the accuracy of the unweighted regression parameters calculated, but analysis of variance is considered sufficiently robust to be still reliable here.

13. I thank Miss Barbara Z. Thoma for technical assistance, Harold A. Montgomery for the construction of the centrifuge, Delbert C. McCune for helpful suggestions regarding the presentation of the results, and the Harvard Forest for a place to prepare the manuscript. Supported by NSF grant 18482.

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Antibodies to Bradykinin and Angiotensin: A Use of Carbodiimides in Immunology

Abstract. *Antibodies to bradykinin and angiotensin have been produced in rabbits by the use of conjugates containing albumin and the hapten, covalently bound. The use of water-soluble carbodiimide reagents provided an easy and rapid method of synthesis of the antigenic conjugates.*

Formation of antibodies to substances of low molecular weight can be stimulated by injecting compounds containing the small molecule conjugated to proteins. Conjugation has usually been achieved by diazotization or other organic syntheses (*1*). We have used water-soluble carbodiimides (*2, 3*), recently developed coupling reagents, to synthesize immunogenic conjugates of protein and biologically active small polypeptides.

Carbodiimides can couple compounds containing many types of functional groups, including carboxylic acids, amines, phosphates, alcohols, and thiols, with the formation of amides, esters, and so forth (*4, 5*). The coupling probably proceeds in at least two steps, as illustrated in reactions *A* and *B* of Fig. 1, for the postulated coupling of hapten to protein through an amide linkage (*4, 6*).

Carbodiimides also can add to carboxylic acids, and, by rearrangement, form a stable *N*-substituted urea. This is illustrated in reactions *A* and *C* of Fig. 1. This addition would yield, in the case of albumin, a substituted urea bound to carboxyl groups of the protein.

It is likely that both products, the hapten-protein complex and the substituted urea-protein complex, were formed during synthesis of the immunogens described in this report. Thus, two "foreign" antigenic determinants were added to the carrier protein, and antibodies to both might have been formed. To minimize the complications of antibodies to the substituted urea, two different carbodiimides were used. The animals were immunized with a hapten-protein conjugate synthesized by the use of one carbodiimide, and their serums were tested with conjugates synthesized by the second carbodiimide.

The two carbodiimides (Fig. 2) were reagent I, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride ("Ethyl CDI") (*7*), and reagent II, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluenesulfonate ("Morpho CDI") (*8*). The carrier protein was rabbit serum albumin (RSA), and the haptens were synthetic bradykinin and synthetic angiotensin (*9*).

Approximately 10 mg of rabbit serum albumin and 20 mg of bradykinin (or other hapten) were dissolved together in 0.5 ml of water. To this mixture was added 0.25 ml of water containing 100 to 200 mg of freshly dissolved carbodiimide reagent I or II. The reaction was permitted to proceed with gentle agitation at room temperature for 5 to 30 minutes. The unadjusted *p*H of the reaction mixture was 6 to 8. The reaction was terminated by dialysis against water for 24 hours. Indirect evidence of successful conjugation of the reactants to albumin was sometimes provided by the formation of precipitates or colloidal suspensions, probably caused by changes in the solubility of albumin when substituents were added. However, formation of visible precipitates did not always follow the formation of conjugates. When precipitates formed, the granular and soluble materials were used together for immunization.

Amino acid analysis of the antigen conjugate of bradykinin and albumin for lysine, histidine, and arginine was performed by the procedure outlined by Spackman, Stein and Moore (*10*). The results are summarized in Table 1. Since

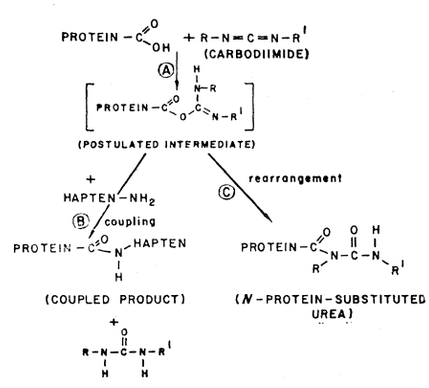

Fig. 1. Probable mechanisms of protein-hapten-carbodiimide reactions.

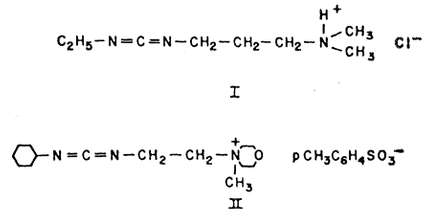

Fig. 2. Structural formulas of two water-soluble carbodiimides.

each molecule of bradykinin contains two arginine residues, but no histidine or lysine, the presence of bradykinin in a protein conjugate is indicated by an excess of arginine to histidine or to lysine as compared to the unconjugated protein. Two such conjugates, the products of separate syntheses, contained 12 moles of bradykinin per mole of albu-

Table 1. Calculation of the amount of bradykinin conjugated to albumin by reaction with carbodiimide reagent. Calculated moles of bradykinin per mole of complex was 12. (Bradykinin contains two residues of arginine but no lysine or histidine.)

Specimen	Arg / Lys*	Arg / His*	Molar excess of arginine in complex
	Experiment 1		
Rabbit serum albumin†	21	19	
Albumin-bradykinin complex	44	44	23–25
	Experiment 2		
Rabbit serum albumin†	21	21	
Albumin-bradykinin complex	45	45	24

* Native rabbit serum albumin contained 55 lysine residues and 21 histidine residues per mole. Bradykinin content calculated from arginine : lysine and arginine : histidine ratios.
† Albumin treated with "Ethyl CDI" in absence of bradykinin.