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Sexual Differentiation in Hydra

Control by Carbon Dioxide Tension

W. F. Loomis

The chemical nature of the stimuli that control cellular differentiation may be approached with advantage in *Hydra*, for it has been shown that these animals do not differentiate sexually in response to an internal stimulus that is part of their life cycle but rather in response to an external stimulus that is controlled by the environmental conditions under which they are cultured. Although previous attempts to define the responsible variable have been unsuccessful, factors such as crowding, stagnation, nutrition, and temperature have been shown to affect the process. A few years ago we reported that *Hydra* differentiate sexually when cultured under crowded conditions such that their oxygen tension was reduced to about 70 percent saturation with air (*1*). Quantitative study of this phenomenon was carried out with the aid of a new and rapid method for determining dissolved oxygen (*2*).

Effect of Surface/Volume Ratio

In the experiment shown in Table 1, four otherwise identical cultures of *Hydra* were grown in differently shaped containers so that the depth of the four cultures and their surface/volume ratios varied progressively. Each culture consisted of 25 *Hydra littoralis* in 25 milliliters of 70-milligram-per-liter $CaCl_2$ and 100 milligram-per-liter $NaHCO_3$. The animals were grown in a beaker and three sizes of petri dishes with internal diameters of 4.8, 6, 9, and 15 centimeters, respectively. Each culture was fed for 30 minutes daily with an excess of brine shrimp larvae (*3*), following which it was rinsed with clean culture solution to remove all uningested brine shrimp and left at 25°C until the following day. A constant degree of crowding was maintained by the daily removal of all newly detached buds.

The results of this experiment demonstrated that, although sexual differentiation and reduced oxygen tension were parallel phenomena, they did not develop proportionally. It appeared likely, therefore, that some other volatile factor, besides oxygen, was the inducing variable. This conclusion was confirmed by the finding that artificial reduction of the oxygen tension did not induce sexual differentiation in *Hydra*.

Subsequent experiments demonstrated that *Hydra* differentiate sexually in response to an unidentified gas given off by the animals themselves (*4*). The rate at which this gas was secreted was found to depend on both temperature and nutrition, being especially high during periods of active digestion. Its rate of accumulation was found to vary with the depth of the water as well as with the degree of crowding and stagnation within the culture. The gas was highly volatile, for brief aeration prevented sexual differentiation from occurring in cultures that otherwise would turn sexual. Its solubility coefficient was of the order of 1, for the gas could be transmitted to the air phase and back into clean water in sufficient quantity so that it still induced sexual differentiation (Table 2).

Air Bridge Experiment

In the air-bridge experiment (Table 2), the sex-inducing gas present in the culture water of crowded *Hydra* was transferred to an air phase and back into clean water by the following technique. Twenty milliliters of "used" culture water, obtained each afternoon from the two cultures described below, were drawn into a 25-milliliter syringe and shaken with 5 cubic centimeters of air for 30 seconds. The air phase alone was then transferred to another syringe, where it was shaken with 10 milliliters of clean water. This treated sample of clean water was given to one culture, while a similar sample of untreated clean culture water was given to the control. A constant degree of crowding was maintained by the daily removal of all newly detached buds. In this experiment, each culture consisted of 10 *Hydra littoralis* in 10 milliliters of 100-milligram-per-liter $CaCl_2$, 125 milligram-per-liter $NaHCO_3$, and 12 milligram-per-liter disodium ethylenediaminetetraacetate (Versene) brought to *p*H 8.0 with NaOH. Both cultures were contained in 15-milliliter beakers and fed and cleaned daily as described in the preceding section. In addition, both cultures received a second afternoon rinse about 5 hours after their daily feeding, at which time the "air-bridge" vessel received the treated water, while the control vessel did not.

Since this experiment indicated that an active gas was present in samples of air equilibrated with "used" culture water, a concentrated sample of this gas was prepared and subjected to analysis by infrared spectrophotometry, mass spectrography (*5*), and gas-liquid partition chromatography. It was found that, within the limits of these instruments, no gases were present other than carbon dioxide, oxygen, nitrogen, and argon, the carbon dioxide concentration being increased and the oxygen concentration decreased relative to their concentrations

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in normal air. Since *Hydra* actively generate CO_2, especially during periods of digestive activity (*6*), and in amounts proportional to their population density, it was suggested that an increased partial pressure of the unhydrated gas CO_2 ("pCO_2") might be the inducing variable (*7*).

A convenient method for determination of pCO_2 was devised. Ten milliliters of the solution to be tested was drawn up into a 20-milliliter syringe containing 10 milliliters of CO_2-free air. The syringe was shaken for 30 seconds to equilibrate the air and water phases, following which the percentage of CO_2 in the gas phase was accurately measured in an improved Henderson-Haldane gas analysis apparatus (*8*). The pCO_2 of the original sample was obtained by multiplying the percentage of CO_2 found in the gas analysis by $(1 + 1/\alpha)$, where α is the absorption coefficient of CO_2 in water at the temperature of the experiment (20°C).

By using this method, a study was made of the lability of solutions whose pCO_2 had been increased by the injection of water previously shaken with various concentrations of CO_2 gas. It was found that brief aeration, decanting, filtering, and so forth rapidly reduced the pCO_2 of such solutions to that of the surrounding air. Furthermore, it was found that undisturbed vessels such as those described in the next section lost their increased content of CO_2 gas at a logarithmic rate whose half-life was approximately 3 hours. Effective elevation of the pCO_2 within such culture vessels therefore demanded twice-daily injection of a solution high in pCO_2. Using this method, it was found that *Hydra* differentiated sexually in cultures whose average pCO_2 had been increased for 10 consecutive days.

Table 1. Percentage of sexual forms and oxygen tension in cultures of differing surface/volume ratio.

Depth (mm)	Oxygen tension (mg/lit)	Percentage of sexual forms after 10 days
30	7.3	100
10	8.4	100
5	8.6	48
2½	8.7	0

Table 2. Air-bridge experiment, in which the sex-inducing gas present in the culture water of *Hydra* was transferred to an air phase and back into clean water.

Day	Percentage of sexual forms	
	Air-bridge	Control
0	100	100
2	100	100
4	80	50
6	50	0
8	80	0
10	90	0
12	80	0
14	60	0
16	50	0
18	100	0
20	100	0

Control by pCO_2

Each culture in the experiment on pCO_2 control of sexual differentiation (Table 3) consisted of 10 asexual *Hydra littoralis* grown in 15 milliliters of 50 milligram-per-liter $CaCl_2$, 100 milligram-per-liter $NaHCO_3$, and 50 milligram-per-liter Versene. Each culture was contained in a 15-milliliter beaker and fed and rinsed twice daily as described in the preceding section. The culture solution was shaken three times before use with a large excess of 100-percent oxygen, yielding a control solution whose pCO_2 was 0.00 percent of an atmosphere (*9*). Water with an initial pCO_2 of 8.35 percent of an atmosphere was prepared twice daily by shaking 20 milliliters of the control solution with 100 milliliters of 10-percent CO_2 and 90-percent O_2 in a large syringe for 1 minute. Graded amounts of this pCO_2-rich water were injected twice a day into the experimental cultures as indicated in Table 3. Care was taken to place the tip of the No. 18 needle as far under water as possible and to inject this pCO_2-rich water with a minimum of aeration. A constant degree of crowding was maintained in the cultures by the daily removal of all newly detached buds.

This experiment demonstrates that sexual differentiation may be induced in *Hydra* by artificially increasing the pCO_2 of the water in which they are cultured. It confirms the fact that reduced oxygen tension is not necessary for sexual differentiation to occur, for the oxygen tension in this experiment was nearly 5 times higher than that of water saturated with air. Finally, it shows that the effects of the sex-inducing gas naturally produced by *Hydra* (air-bridge experiment) can be duplicated by using chemically pure carbon dioxide gas (Matheson).

Since increasing levels of pCO_2 progressively decrease the *p*H of alkaline solutions, other experiments were conducted in which *Hydra* were cultured in solutions buffered with tris(hydroxymethyl)aminomethane. It was found that cultures of *Hydra* remain asexual regardless of *p*H when the animals are grown under uncrowded or aerated conditions. Since the experiments described in Tables 1 and 2 demonstrate that the bicarbonate ion per se is unable to induce sexual differentiation in *Hydra*, it was concluded that the labile variable involved is pCO_2.

Carbon Dioxide Tension

The fact that pCO_2, and not total CO_2, reversibly (*10*) induces sexual differentiation in *Hydra* emphasizes the difference between these two variables. The remainder of this article is devoted to a discussion of these differences, together with a brief survey of some other biological phenomena known or suspected to be specifically dependent on pCO_2.

In physical chemistry, pCO_2 is defined as the partial pressure of the unhydrated gas CO_2 physically dissolved in water. It differs from total CO_2 in that it is concerned with only one of the four forms of dissolved CO_2 (CO_2, H_2CO_3, HCO_3^-, and CO_3^{--}). Since the partial pressure of a gas is proportional to its percentage composition, the pCO_2 of normal air is 0.03 percent of an atmosphere. Water in equilibrium with such air has a pCO_2 of 0.03 percent of an atmosphere regardless of *p*H and bicarbonate concentration. Higher values of pCO_2 may exist (i) in closed systems equilibrated with air containing higher concentrations of CO_2, and (ii) in open solutions that are *not* in equilibrium with the surrounding air and within which CO_2 is steadily generated. The level of pCO_2 within such open cultures depends on the dynamic equilibrium existing between the relative rates of CO_2 generation on the one hand and CO_2 release from the surface on the other.

Analytically, pCO_2 may be determined by finding the concentration of CO_2 gas that is in equilibrium with the solution to be tested (*11*), as in the method described in this article. Methods based on *p*H determinations have been described (*12*), but they require additional measurements of total base. Operationally, therefore, pCO_2 may be distinguished from total CO_2 by determining the amount of CO_2 released from a solution by (i) simple shaking with air or (ii) the addition of acid.

Factors Affecting Carbon Dioxide Tension

The fact that pCO_2 values above 0.03 percent of an atmosphere are possible only in open cultures which are *not* in equilibrium with the air makes this variable peculiarly labile. Any operation that equilibrates the culture with the surrounding air reduces its pCO_2. Thus, such seemingly gentle operations as stirring, decanting, and filtering can rapidly lower the pCO_2 of a solution. In addi-

Table 3. Control of sexual differentiation in *Hydra* by pCO_2

Vessel	1	2	3	4	5	6	7	8
Culture water shaken with 100 percent O_2 (ml)	15		14		10		5	
Culture water shaken with 10 percent CO_2 and 90 percent O_2 (ml)	0		1		5		10	
Initial pCO_2	0.0%		0.6%		2.8%		5.6%	
Day	Percentage of sexual forms							
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	10	0	0	0
9	0	0	10	30	70	70	70	60
10	0	0	60	50	100	100	100	100
11	0	0	70	60	100	100	100	100
12	0	0	100	60	100	100	100	100
13	0	0	100	70	100	100	100	100

tion, any variable that affects the rate of CO_2 generation within a solution affects its pCO_2. Increasing the number, size, rate of respiration, respiratory quotient, and so forth of any animals within a culture tends to increase its pCO_2, while the opposite is true of photosynthesizing plants. Since CO_2 may be generated indirectly by the release of acid groups in a bicarbonate solution, both glycolysis and respiration are capable at times of affecting pCO_2. In addition, any increase in the concentration of CO_2 in the air affects the pCO_2 of solutions open to that air, so that such apparently unrelated events as lighting Bunsen burners, working within a small, unventilated culture room, and so forth may affect the pCO_2 of open cultures (*13*).

The subtle character of these various factors combines to make pCO_2 one of the most labile and neglected of all biological variables (*14*). Yet increased levels of pCO_2 have been shown to affect the respiratory rate and respiratory quotient of living cells, as well as the process of aerobic glycolysis (*15*). Krogh has shown that CO_2 gas differs markedly from carbonic acid in that it can penetrate cell walls more easily than any other known substance, including water (*16*). Jacobs has demonstrated that this highly liposoluble gas can penetrate cell membranes selectively, thus producing intracellular acidity even in alkaline solutions (*17*).

The pCO_2 of a solution therefore differs from its content of total CO_2 as much as the pH of a solution differs from its content of total acid. Just as "acidity" today is resolved into two components—the concentration of the hydrogen ion, and the quantity of acid potentially capable of furnishing additional hydrogen ions—so "dissolved carbon dioxide" is resolvable into two components—the concentration of unhydrated CO_2 molecules, and the quantity of bicarbonates and carbonates potentially capable of furnishing additional CO_2 molecules. In a given solution, Henry's law states that the concentration of CO_2 gas is directly proportional to its pCO_2:

$$[CO_2] = \alpha\, pCO_2$$

where α is the solubility of CO_2 gas in that solution at any given temperature (*18*). Finally, it is known that the amount of dissolved CO_2 gas is approximately 1000 times the amount of H_2CO_3 present in a solution (*19*) and that the solubility coefficient of CO_2 in water is approximately 1 (*20*).

Known Instances of Control of Biological Phenomena by pCO_2

Reviewing briefly some cases where pCO_2 has been shown to control biological phenomena, it need only be mentioned that the respiratory center of the brain is highly sensitive to this variable (*21*). Powers has pointed out that lung-breathing animals have the dual advantage of living in an environment of fixed CO_2 composition and of possessing closed alveolar spaces within which the pCO_2 of the air may be closely regulated (*22*). He stated that fish, for example, are very sensitive to the pCO_2 of water, for their gills are essentially open systems and hence highly responsive to the variable pCO_2 found at various depths in lakes and other bodies of water. Powers and others have held that pCO_2 is a prime ecological variable, capable of controlling both the habitats of different species of fish as well as the migratory movements of salmon (*23*). Davidson has recorded a striking instance of catastrophic death in fish, apparently produced by a sudden increase in pCO_2 (*24*).

The process of photosynthesis is another biological phenomenon that is sensitive to pCO_2. Aquatic plants and algae, for example, selectively absorb free CO_2 molecules rather than the almost totally impermeable bicarbonate ions (*25*). Thus, Emerson and Green found that at optimal levels of pCO_2 (about 0.1 percent of an atmosphere) "neither hydrogen ion nor bicarbonate ion concentration influences the rate of photosynthesis between pH 4.6 and 8.9" (*26*).

Possible Instances of Control of Biological Phenomena by pCO_2

Went and others have shown that both vegetative growth and floral differentiation in plants are highly dependent on the relative duration and respective temperatures of their day and night cycles (*27*). Since the primary day reaction is the CO_2-utilizing reaction of photosynthesis and the primary night reaction the CO_2-generating process of respiration, it appears possible that these effects are mediated by changes in the pCO_2 of the tissues of the plant. Crocker has shown, for example, that increased concentrations of CO_2 in the air regulate plant growth in a manner strikingly similar to that of ethylene chlorohydrin (*28*).

Several other obscure biological phenomena may well depend on this curiously labile variable. As in *Hydra,* sexual differentiation in Protista, myxamoebae, *Bonellia, Crepidula, Daphnia,* and other genera (*29*) would appear to depend on critically increased levels of pCO_2. Banta, for example, showed that the stimulus to sexual maturation in Daphnia was a nonspecific gas given off by many types of animals. Ketchell and Williams found that tissue cultures of Cecropia larva spermatocytes gave off a gas that induces spermatid differentiation when it is present in sufficient concentration. Whitaker has described the group effects long observed in germinating *Fucus* eggs as dependent on "a common action of hydrogen ions and CO_2" (*30*). Cooper demonstrated that *Rana pipiens* eggs gave off a "hatching secretion" that was inactivated on standing in the cold, as well as by simply passing it through a filter (*31*). Clowes described a sperm-stimulating gas derived from marine eggs (*32*), while Cook and Elvidge recently showed that a similar sperm-stimulating gas was given off nonspecifically by several species of Fucaceae eggs. He showed that this gas was chloroform-soluble and able to penetrate even

the thick walls of oogonia (*33*). Kisimoto found that crowding the larvae of the plant hopper *Nilaparvata* in the bottom of test tubes differentially produced flying forms of this insect, the effect varying with the degree of crowding of the larvae (*34*).

The extensive work of Child has shown that differentiation and morphogenesis are intimately affected by respiratory and metabolic gradients (*35*). It need only be mentioned here that all such gradients are, *ipso facto,* gradients of pCO_2. Bellamy observed that adding $5 \times 10^{-5} N$ HCl to the water in which frog eggs were developing produced "a very marked acceleration of development. Both Professor Child and myself have obtained these acceleration forms independently and repeatedly" (*36*). Bellamy's striking results, supported by illustrations, may well be the result of a critical elevation of pCO_2, for addition of dilute acid to the carbonate-containing well water used in his experiments would raise the pCO_2 in unequilibrated solutions. Merwin showed that the rate of development of frog eggs was stimulated by crowding various numbers of eggs together, as well as by exposing them to air containing 0.3 percent CO_2 (*37*). Trinkaus and Drake reported that CO_2 in the gas phase stimulated differentiation in *Fundulus* embryos (*38*), while Spratt concluded from similar observations in chick embryos that "it is possible that carbon dioxide may be as fundamental a requirement of the early embryo as oxygen" (*39*). Rather than being merely a waste product of metabolism, therefore, it appears that CO_2 is capable of regulating cellular differentiation. Since the concentration of this penetrating gas is automatically highest toward the center of a mass of tissue, gradients exist from within outward that are a function of the whole, reforming whenever part of a tissue is cut away. Since these field characteristics are also those of the developing embryo, it appears possible that pCO_2 gradients, generated by the cells themselves, may be one of the important variables controlling embryologic development.

Moen has shown that single cells will not grow *in vitro* unless they receive an "influence" from neighboring cells that need not be in contact with them (*40*). Earle and his associates were able to grow single cells in isolation by sealing them in capillary tubes filled with air that was enriched with 5 percent CO_2 (*41*). Recently Puck has shown that single cells can be grown in media in which a "short-lived, diffusible factor" is generated by x-irradiated feeder cells (incapable of multiplication themselves) placed in close juxtaposition to the single multiplying cell (*42*). In all of these cases, it would appear possible that pCO_2 was the mysteriously labile variable. Certainly the pCO_2 within a tissue culture is generally high, for the usually alkaline culture medium is equilibrated with approximately 4 percent CO_2 in the usual routine of tissue culture (*43*). Although the rationale for equilibrating the medium with CO_2 gas is universally stated to be the establishment of a correct pH, it is clear that there are easier ways of doing this today and that this particular method may well be required for the establishment of a correct level of pCO_2 (*44*).

Inhibition of Growth by High Levels of pCO_2

The stimulating effects of low levels of pCO_2 on growth and differentiation contrast with the inhibitory effects of high levels. Smith and Clowes, for example, found that cell division in marine eggs could be *reversibly* arrested by sufficient elevation of the pCO_2 (*45*). Haywood and Root studied these effects quantitatively, finding that levels of pCO_2 above 5 percent of an atmosphere markedly inhibited both respiration and cell division, the latter being completely arrested at about 16 percent of an atmosphere (*46*). Since the pCO_2 of human tissues is always above 5.3 percent of an atmosphere (its level in arterial blood, *21*), it appears that high levels of pCO_2 may be the primary reason for the arrest of cell division in most adult mammalian tissues.

Possible Relation to Cancer

It has been repeatedly observed that normal cells grown for long periods of time in tissue culture have a tendency to turn cancerous (*47*). In fact, the progressively anaerobic environment of a tissue culture apparently "provides just the conditions needed to transform the metabolism of normal epithelial cells to that of malignant cells" (*48*). To date, the only operative variable that has been suggested has been that of intermittent anoxia (*49*). Just as likely, perhaps, is the fact that the gradually increasing pCO_2 within a tissue culture provides an environment favorable to the growth of any mutant cell that is even partially resistant to the inhibitory effect of high levels of pCO_2. Since variation in resistance to high levels of pCO_2 is known to occur both between species (*23*) and within a single species (*50*), it may be that resistance to pCO_2 inhibition can appear in cells grown for long periods of time in an environment where pCO_2 is the limiting variable. Certainly any inherited resistance to pCO_2 inhibition, however partial, would result in the selective growth of a resistant cell strain. Indeed, Mottram has suggested that very high levels of pCO_2 may even produce somatic mutations, for he observed that levels of pCO_2 of about 10 to 20 percent of an atmosphere produced abnormal mitotic figures very similar to those produced by radiation. He concluded that "a localized increase of the carbon dioxide tension in the tissues, due to a diminished blood supply, may be an important factor in the cancerous change of cells and may even be the factor common to many known 'causes' of cancer" (*51*).

Whether malignant growth actually results from the *de novo* appearance of a partially pCO_2-resistant cell strain selectively capable of growing in an environment whose pCO_2 is sufficiently high to inhibit the growth of its normal cell neighbors, only further work can determine.

Conclusion

It has been found that sexual differentiation may be reversibly induced in *Hydra* by measures that control the pCO_2 of their aqueous environment. The marked differences between pCO_2, or the partial pressure of dissolved CO_2 gas, and total CO_2 as usually measured are discussed both chemically and biologically. In general, it appears that the level of pCO_2 in the environment of a living cell is one of the most labile and neglected of all biological variables, yet one that is capable of regulating both the rate of cell division and the process of differentiation.

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News of Science

Sputnik

The U.S.S.R. launched the first earth satellite, *Sputnik,* on Friday, 4 October. In the United States a group of participants in an International Geophysical Year conference was being entertained at the Russian Embassy when the news was announced. Lloyd Berkner, American IGY representative who is the reporter on earth satellites for a special IGY committee, interrupted the embassy festivities to commend the Soviet scientists for their historic achievement.

In a published statement, Joseph Kaplan, chairman of the United States National Committee for IGY, said:

"I am amazed, [given] the short time which they had to plan—obviously not any longer than we had. I think it was a remarkable achievement on their part. From the point of view of international cooperation the important thing is that a satellite has been launched. They did it and did it first."

P. H. Wyckoff, another member of the United States IGY committee, commented: "We are all elated that it is up there."

The text of the first Soviet report on the *Sputnik* included the following statements:

"According to calculations . . . the satellite will revolve at heights of up to 900 kilometers [500 miles], making one complete revolution [at 18,000 miles an hour] in one hour 35 minutes, the angle of its orbit to the equatorial plane being 65 degrees. . . .

"The satellite is in the form of a sphere with a diameter of 58 centimeters [about 22 inches] and weighs 83.6 kilograms [about 184 pounds]. It carries two radio transmitters emitting continuously signals of 20.005 and 40.002 megacycles frequency [about 15 and 7.5 meters wavelength, respectively]. The transmitters are powerful enough to insure good reception by wide numbers of amateur radio operators.

"The signals are sent in the form of telegraph messages lasting some 0.3 seconds with a pause of the same duration in between.

"The signal on one frequency is sent during the pause in the transmission on another frequency. . . .

"The Soviet Union proposes to send up several more artificial satellites during the International Geophysical Year. These will be bigger and heavier and will help to carry out an extensive program of scientific research. . . ."

Three days after the launching, Moscow radio reported that:

"The carrier rocket is just now revolving around the earth at approximately the same altitude—560 miles—as the satellite. The distance between them is about 625 miles. The distance will grow."

On the morning of 8 October, Moscow radio again issued a report, this time a warning that the satellite power supply was almost expended, that its batteries had been expected to last "only a few days." Later in the day the signals were lost for a time, but reappeared as strongly as ever after a lapse of several hours.

On Wednesday, 9 October, the Soviet Union's newspaper *Pravda* published for the first time details of the satellite and its rocket:

"The successful launching of the man-made moon, has fully confirmed the correctness of the calculations . . . in designing the carrier rocket and the satellite.

"The satellite was placed in the nose of the carrier rocket and shielded by a protective cone. The rocket was fired vertically. Moving around the world now is not only the baby moon and the carrier-rocket, but the protective cone as well. . . .

"Inasmuch as the time between the jettisoning of the cone and the detachment of the satellite was not great, the rocket and the cone were comparatively near the satellite for some time. . . .

"Then, due to the difference in rotation periods arising both from the relative speeds at the time of detachment and from the varying degrees of atmospheric resistance, the three objects moved apart and in their further rotation could be spotted over absolutely different points of the world at the one and same moment.

"The altitude of the satellite varies. It changes periodically, reaching the highest point of approximately 1000 kilometers [600 miles].

"At present the orbit's perihelion [low-