We describe here the application of the ion shower milling machine to remove the cell membrane of the single isolated neuron. We also measured the content of calcium ions in the normal neuron and that during bursting activity induced by pentylenetetrazole (PTZ) both for the whole cell and for the cell with the membrane removed.

We used the D neurons of the subesophageal ganglion of the Japanese land snail, *Euhadra peliomphala*, in the active season. Two groups of dissected ganglia were prepared, one of which was incubated in normal snail Ringer solution and the other in snail Ringer solution containing $5 \times 10^{-2} M$ PTZ. After 10 minutes of incubation, both groups were quickly frozen by being dipped in a liquid Freon 12 pool surrounded by solid Freon 12 cooled by liquid nitrogen and freeze-dried without passing through a liquid phase at a temperature below −35°C. We also made some freeze-dried specimens at a temperature below −80°C, but the results were the same. The D neurons were dissected from the freeze-dried ganglion with fine forceps under a binocular microscope. Each freeze-dried neuron was cut into two hemispherical shapes under the microscope, and half of each group was placed on the ion shower stage. The other half was not etched.

We used the Kaufman type ion shower milling machine (Elionix ISM-S) and carried out ion etching for 3 minutes in argon at a pressure of $6 \times 10^{-5}$ torr. The ion-accelerating voltage was 500 V, and the ion current was 200 $\mu$A. In order that the surface of the neuron be etched evenly, the sample stage was inclined to 45° and rotated both around the vertical axis and the stage axis during ion etching. The etched and nonetched D neurons were weighed with a fish-pole balance placed in a transparent plastic box with manipulating windows which was located in a room kept at a constant temperature (20°C) and constant humidity (40 percent) according to the microtechnique of Lowry and Passonneau (*2*). The mean weight of a freeze-dried D neuron was 93.6 ± 9.8 ng (mean ± standard deviation, $N = 31$). The weight of an ion-etched neuron was almost the same as that of a nonetched neuron.

Figure 1 shows the electron micrograph of the cell surface of a freeze-dried normal neuron (A) and an ion-etched neuron (B) prepared by the conventional Epon embedding method. The ultrastructure of the ion-etched neuron was not as well preserved as that of the normal neuron because of freeze-drying but the cell membrane was completely removed. Figure 2 shows the calcium content of normal cells and ion-etched cells with the membranes removed measured by a flameless atomic analyzer (JEOL JAA-7000; sensitivity, 0.5 part per trillion) for both normal and PTZ-treated cells. The difference between the whole cell calcium content of a neuron dipped into normal snail Ringer and that of a neuron dipped into calcium-free Ringer was negligible. This demonstrates that more than one-fifth of the whole cell calcium is distributed within the membrane area. In the case of PTZ-treated cells, the calcium content of the cell membrane area was considerably increased compared with normal cells, as shown in Fig. 2 (*3*).

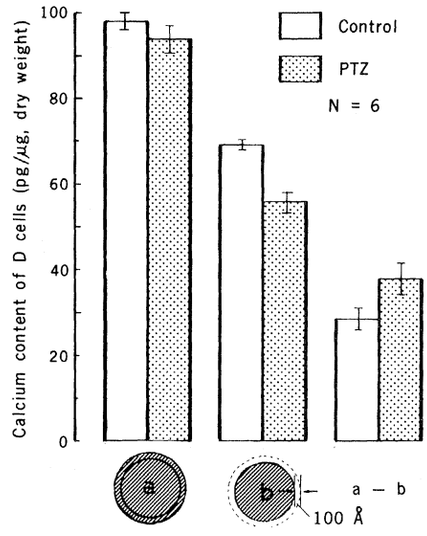


Fig. 2. Calcium content of normal and PTZ-treated neurons. The pair of bars at the left shows the whole cell calcium content, the middle bars show the calcium content after removal of the cell membrane area (about 100 Å), and the bars at the right are calculated values of the calcium content of the cell membrane area.

These findings demonstrate that the calcium accumulates in the cell membrane area during bursting activity induced by PTZ. The ion shower milling machine provides a promising technique for cell membrane analysis.

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**References and Notes**

1. S. M. Lewis, J. S. Osborn, P. R. Stuart, *Nature (London)* **220**, 614 (1968).
2. O. H. Lowry and J. V. Passonneau, *Flexible System of Enzymatic Analysis* (Academic Press, New York, 1972).
3. A question may arise about the possibility of the redistribution of ions or molecules during the ion shower milling procedure. We examined this possibility by carrying out the following experiment. Two groups of several cells each were prepared: one was used as a control, and the other was ion-showered under the same conditions as we used in this experiment. The distribution pattern of calcium on the inner surface of the hemispherically cut cells of both groups was analyzed by computer-controlled ion distribution mapping (*4*). No change in the distribution pattern was found. One may question whether a change in the calcium distribution may occur during the freeze-drying. If the ion distribution change came from freeze-drying, the D cells and I cells would show the same change tendency under the same procedure. However, after the same freeze-drying procedure the calcium distribution map of the PTZ-treated D and I cells showed completely different distribution patterns, as described in (*4*).
4. E. Sugaya and M. Onozuka, *Science* **200**, 797 (1978).
5. We thank M. Hotta for help in manipulating the ion shower milling machine.

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## The Pineal Gland: A Biological Clock in vitro

Abstract. *Circadian rhythmicity was studied by following the course of* N-*acetyltransferase activity in the pineal glands of chickens in vitro. The results indicate (i) a daily change during day 1 of organ culture in constant dark that was dependent on the time the chickens were killed, (ii) equivocal persistence of the daily change in constant dark during 6 to 7 days of organ culture, (iii) an effect of light, (iv) inhibition by adrenergic agents and cycloheximide, and (v) stimulation by dibutyryl adenosine 3′,5′-monophosphate and related compounds.*

Serotonin *N*-acetyltransferase in the pineal gland of the chicken takes part in the conversion of the indole serotonin to the pineal hormone melatonin (*1*). There are circadian rhythms in melatonin production, and these are believed to be regulated by a circadian rhythm in serotonin *N*-acetyltransferase activity (NAT) (*2*, *3*).

The daily rhythms seen in NAT in the pineal gland are thought to be of special importance for the following reasons. (i) The pineal gland has been established as the source of the circadian rhythm in sparrows by investigators using ablation, transplant, and hormone replacement techniques (*4*); (ii) pineal gland NAT has in vivo a circadian rhythm that is precisely timed and regulated by light and dark (*2*); and (iii) the NAT rhythm has been observed to reach a peak in the dark time in all species of mammals and birds so far studied, irrespective of whether the species was nocturnal or diurnal in behavior (*5*).


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In seeking the source of the daily rhythm in NAT, we began using organ culture to investigate NAT in pineal glands of chickens. Whole pineal glands were obtained for these studies from chicks (*6*) that had been kept on light-dark (LD) schedules of 12 hours of light and 12 hours of darkness from day 1 after hatching. The pineal glands were placed in organ culture (*7*), treated in various ways during culture, harvested from culture, and assayed for NAT (*8*).

In early work with the organ cultures in constant dark we observed that a peak occurred during day 1 of organ culture (Fig. 1a). Some difficulties with reproducing this first peak were resolved when we discovered that the occurrence and height of the peak on day 1 depended on the time of day that the chickens were killed (Fig. 2). Pineal glands of chicks killed late in their subjective light time show a peak coincident with the dark-time projected from the light-dark cycle to which the chicks had been exposed (Fig. 2a). The height of the peak increased when the chickens were killed closer to the time of projected dusk (Fig. 2a). Pineal glands of chicks killed early in the light time did not have a peak during day 1 (Fig. 2b). Pineal glands of chicks killed in the dark time began with high NAT, which declined at approximately the time of projected dawn (Fig. 2c) (*2, 3*). In other experiments we have shown that the nature of the decline of NAT in glands from chickens killed in the dark is dependent on prior photoperiod length (*9*). From these organ culture experiments we conclude that the pineal glands of chickens have a kind of "memory" of the timing of the lighting conditions to which their donors were exposed.

To ascertain whether the timing mechanism for the circadian rhythm that persists in constant dark in NAT could reside entirely within the pineal gland, we placed pineal glands from chickens killed near dusk into organ culture in constant dark for 6 or 7 days (Fig. 1b) (*9*). In both of two experiments there was a peak on day 1 and a "possible" peak on day 2. However, after day 1 of culture, the NAT did not clearly oscillate, nor were the values reproducible between the two experiments. We consider this to be debatable evidence that the pineal glands of chickens are capable of maintaining oscillation in vitro in constant dark.

Since the NAT rhythm is precisely timed in vivo by environmental lighting we examined the responsiveness of the pineal gland to light in vitro. In an organ culture experiment in which glands from chicks killed late in the light time were exposed to lighting changes in culture, NAT was affected by light in culture (Fig. 3). In light-dark there was a sevenfold peak, in constant dark there was a fourfold peak, and in constant light (1400 lux) there was a threefold change that could also be interpreted as a delayed peak (*10*). The avian pineal gland has photoreceptor-like structures (*11*) whose function may be to mediate the light-dark response of NAT in vitro.

Our experiments in vivo indicate that chicken pineal gland rhythms are not wholly autonomous but are responsive to signals from the body and from the eyes: (i) The rapid decrease in NAT produced by exposing chicks to light in their subjective dark time does occur in blinded chickens but is even more rapid when the eyes are intact (*2*). (ii) NAT can be modified by the injection of a number of substances. For example, the dark-time peak can be prevented or delayed by injecting chicks with cycloheximide, isoproterenol, alcohol, histamine, epinephrine, serotonin, pargyline, and L-dopa (*11*). (iii) The superior cervical ganglion may be necessary for the persistence of the rhythm in constant dark but not in light-dark (*3*). The rat pineal gland is precisely regulated by signals originating outside the gland through an adrenergic cyclic AMP (adenosine 3′,5′-monophosphate) mechanism (*13*).

With these facts as a basis, we made pharmacological studies (*10, 14*) of chick-

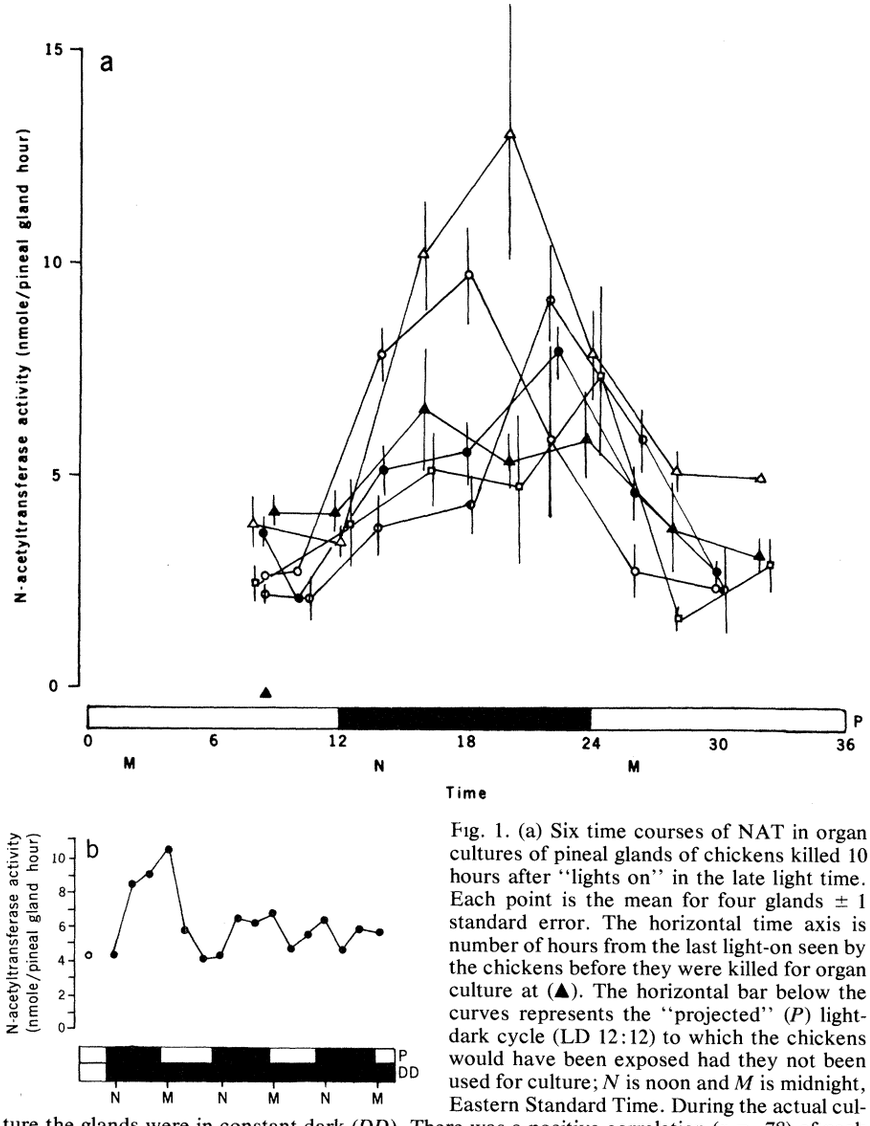



Fig. 1. (a) Six time courses of NAT in organ cultures of pineal glands of chickens killed 10 hours after "lights on" in the late light time. Each point is the mean for four glands ± 1 standard error. The horizontal time axis is number of hours from the last light-on seen by the chickens before they were killed for organ culture at (▲). The horizontal bar below the curves represents the "projected" (*P*) light-dark cycle (LD 12:12) to which the chickens would have been exposed had they not been used for culture; *N* is noon and *M* is midnight, Eastern Standard Time. During the actual culture the glands were in constant dark (*DD*). There was a positive correlation ($r = .78$) of peak attained with the age of the chickens. (b) Time course of NAT in organ cultures of pineal glands of chickens killed 10 hours after lights on in the late light time. The points in the curve are the combined means from two separate experiments (*9*) that ran 6 and 7 days; each point is the average NAT for eight glands. Only the first 3 days of culture are shown here. Otherwise as in (a).

en pineal glands in organ culture. In evaluating pharmacological results for the pineal system, it is necessary to consider the status of the glands at the time of killing (Fig. 2) because a "shift" of the peak may be the result of a given chemical rather than a true stimulation or inhibition. Apparent stimulation can be achieved with dibutyryl cyclic AMP ($10^{-3}M$, 1.7- to 1.8-fold), theophylline ($10^{-3}M$, 2.2- to 2.6-fold), adenosine ($10^{-3}M$, 1.8-fold), adenosine diphosphate (ADP) ($10^{-3}M$, 1.4-fold), and AMP ($10^{-3}M$, 1.8-fold). Apparent inhibition can be obtained with cycloheximide ($10^{-3}M$, 74 percent), isoproterenol ($10^{-4}M$, 70, 33, and 28 percent), norepinephrine ($10^{-4}M$, 65 and 41 percent), and pargyline ($10^{-4}M$, 82 and 69 percent).

The conclusions from these data are that the pineal gland has within itself precise innate timing ability, but that it is also responsive to afferent stimuli.

1) The pineal gland has a "memory" of time in that the NAT response is dependent on the time chicks are killed in a light-dark cycle. Furthermore, when chicks are killed late in their light time or during the dark time, their pineals illustrate timed NAT responses coincident with the projected times of dusk and dawn for one cycle while the glands are in constant dark. The timed peak at day 1 and the timed decline from that peak are unequivocal evidence for timekeeping ability by the pineal gland.

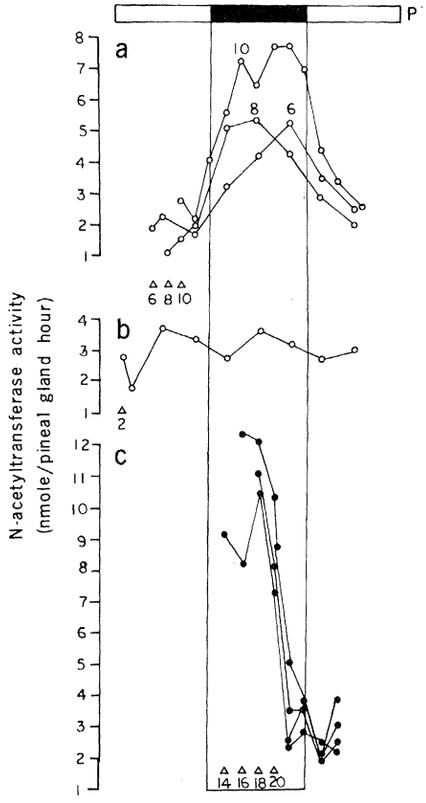
2) The question of whether the pineal gland can, in and of itself, maintain an endogenous oscillation with a circadian period has been addressed by studies in constant dark where we have found a reliable first day peak, a possible second day peak, and not much for the ensuing 4 or 5 days of organ culture. We consider the data to be equivocal regarding an endogenous oscillation because (i) the pineal glands may be simply losing their individual phase relationships, (ii) culture conditions may be inadequate in some way to support persistence of the oscillation, or (iii) the data may reflect a true inability of the gland to maintain the oscillation indefinitely on its own. The fact that killing time affects the resultant peak on day 1 indicates to us that a "buildup" of some capacitating factor is necessary for a daily peak to occur, somewhat like an hourglass (Fig. 2).

3) The chicken pineal gland is directly responsive to light and dark.

4) Pharmacological manipulation of the chick pineal glands in organ culture (supported by injection experiments) reveals pineal responsiveness to chemical stimuli. The data support a possible cyclic AMP involvement in the initiation of NAT, protein synthesis involvement in the initiation of NAT, and adrenergic inhibition of NAT for chicks. However, we have not observed the large stimulatory effects of cyclic AMP and adrenergic agents reported for the rat, and, indeed, the chick adrenergic response seems to be opposite (inhibitory) from that seen in the rat (stimulatory).

The data in vitro match data previously obtained in vivo except that the NAT rhythm persists in constant dark in vivo for at least 2 weeks (*2, 3, 15*).

Our results indicate that pineal gland NAT is a biological clock, a device that measures time, in vertebrates. However, pineal experiments with birds have not readily been verified from one species to the next. In vertebrates there is evidence for other clocks or oscillators located in the hypothalamus-pituitary-adrenal system (*16*). Many organisms lack a pineal gland altogether although indoles are commonly found in both plants and animals. It is not yet clear to what extent the mechanism for timing in the pineal gland relates at a cellular level to other circadian biological clock systems.



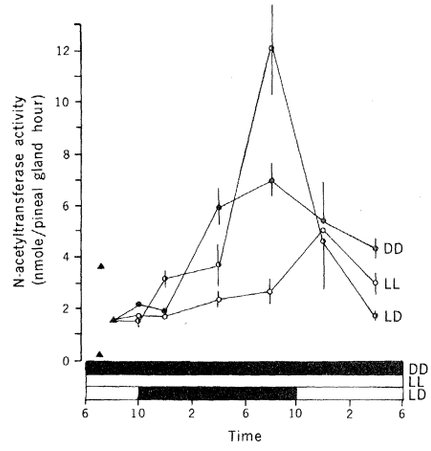




Fig. 2 (left). Time course of NAT in organ cultures of pineal glands of chickens. The horizontal axis is time relative to the projected light-dark cycle (LD 12:12) represented by the 36-hour horizontal bar over the graphs; (△) represents the start time for the cultures. Each curve is the mean time course for cultures started at the time indicated by the △ below the first point on the left of the curve. (○) Pineal glands from the light killed chickens: (●) pineal glands from dark killed chickens. (a) The time course for NAT in organ cultures started late in the light time (6 hours after lights on, two experiments; 8 hours after lights on, one experiment; 10 hours after lights on, six experiments). Each point shown represents the mean for one to six experiments with four glands per experiment (4 to 24 glands). Standard errors have been omitted for visual simplicity but are given in Fig. 1 for six of the cultures. (b) Time course for NAT in organ cultures started early in the light time (2 hours after lights on, two experiments). Otherwise as in (a). (c) The time course for NAT in organ cultures started at four times during the dark time [2, 4, 6, and 8 hours after lights off (*2, 3*)]. Otherwise as in (a). Fig. 3 (right). Time course of NAT in organ cultures of pineal glands of chickens killed in the late light time but cultured under three different lighting conditions. Temperature, measured with a standard laboratory centigrade thermometer, was 37°C in all conditions. The fluorescent light source, a 20-watt Cool White fluorescent bulb, was separated (for insulation) from the cultures by a glass door, plexiglass chamber wall, plastic culture dish, and, in the case of dark by 3-mil black plastic. The horizontal bars below the graphs represent the lighting conditions during culture. The light-dark cycle for the culture was set at the same on-off times as the light-dark cycle to which the chicks were exposed prior to killing. The chicks were 23 days of age when they were killed for the experiment; the cultures were run simultaneously. Otherwise as in Fig. 1.

The data in vitro match data previously obtained in vivo except that the NAT rhythm persists in constant dark in vivo for at least 2 weeks (*2, 3, 15*).

Our results indicate that pineal gland NAT is a biological clock, a device that measures time, in vertebrates. However, pineal experiments with birds have not readily been verified from one species to the next. In vertebrates there is evidence for other clocks or oscillators located in the hypothalamus-pituitary-adrenal system (*16*). Many organisms lack a pineal gland altogether although indoles are commonly found in both plants and animals. It is not yet clear to what extent the mechanism for timing in the pineal gland relates at a cellular level to other circadian biological clock systems.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### References and Notes


1. S. Binkley, S. E. MacBride, D. C. Klein, C. L. Ralph, *Science* **181**, 273 (1973).
2. S. Binkley, C. Ralph, S. MacBride, D. Klein, *Endocrinology* **96**, 848 (1975); S. Binkley and E. Geller, *J. Comp. Physiol.* **99**, 67 (1975); C. L. Ralph, D. Mull, H. Lynch, L. Hedlund, *Endocrinology* **89**, 1361 (1971); C. Ralph, R. Pelham, S. MacBride, D. Reilly, *J. Endocrinol.* **63**, 319 (1974); S. Binkley, J. B. Riebman, K. B. Reilly, *Science* **197**, 1181 (1977).
3. C. Ralph, S. Binkley, S. MacBride, D. Klein, *Endocrinology* **97**, 1373 (1975).

4. S. Gaston and M. Menaker, *Science* **160**, 1125 (1968); S. Binkley, E. Kluth, M. Menaker, *ibid.* **174**, 311 (1971); N. Zimmerman and M. Menaker, *ibid.* **190**, 477 (1975); N. Zimmerman, thesis, University of Texas, Austin (1976); F. W. Turek, J. P. McMillan, M. Menaker, *Science* **194**, 1441 (1976).
5. J. Stephens and S. Binkley, *Experientia*, in press.
6. Day-old chicks (White Leghorn, *Gallus domesticus*) were obtained from George Shaw (West Chester, Pa.). The chicks were housed in heated brooders in experimental rooms in which the lighting schedules could be programmed with timers. The light intensity during the light time was 300 lux (measured with a photodiode-voltmeter system, Ultra-detector, Keithley) and during the dark time was 0. Light was provided by Cool White fluorescent lamps (Sylvania F 30 T12 Lifeline in ceiling fixtures). Groups of chicks of a single age were killed and used for experiments; the ages of the groups used were from 21 to 39 days.
7. D. Klein, in *The Thyroid and Biogenic Amines*, I. Rall and J. Kopin, Eds. (North-Holland, Amsterdam, 1972), pp. 550-568; our cultures were done with Falcon plastic organ culture dishes in each of which a Millipore filter was supported on a 1-ml well of culture medium; we placed two pineal glands on each Millipore filter so that the glands were at the gas-liquid interface. Glands were harvested from culture and frozen in plastic dishes cooled by Dry Ice.
8. D. C. Klein and J. L. Weller, *Science* **169**, 1093 (1970); we use the assay with serotonin as a substrate for chickens. We have observed that higher NAT seems to result with the serotonin-based assay than with the tryptamine-based assay (*4*). We express NAT as nanomoles per pineal gland per hour, as a physiological measure of the gland's ability to produce melatonin. In vivo light time NAT was 1.8 ± 0.2 nmole per gland per hour from glands weighing 1.9 ± 0.3 mg with 0.083 ± 0.007 mg of soluble protein per gland (21.4 ± 2.1 μmole per milligram of protein per hour); in vivo dark time NAT was 17.2 ± 1.0 nmole per gland per hour from glands weighing 1.6 ± 0.1 mg with 0.077 ± 0.044 mg of soluble protein per gland (161 μmole per milligram of protein per hour). Dark time NAT does not correlate with gland weight or protein ($r = -.05$ and .09, respectively); light time NAT does correlate with gland weight and protein ($r = .84$ and .68, respectively). Tryptamine does not compete with serotonin when it is added to the reaction mixture for the assay with chick glands.
9. J. Riebman and S. Binkley, *Comp. Biochem. Physiol.*, in press.
10. S. D. Wainwright and L. K. Wainwright, *Can. J. Biochem.* **56**, 685 (1978).
11. P. Wight, in *Physiology and Biochemistry of the Domestic Fowl*, D. Bell and T. Freeman, Eds. (Academic Press, London, 1971), vol. 1, p. 549.
12. S. Binkley, J. Riebman, K. Reilly, *Comp. Biochem. Physiol. C* **59**, 165 (1978).
13. D. C. Klein and J. L. Weller, *Science* **177**, 532 (1972); D. C. Klein, G. R. Berg, J. Weller, *ibid.* **168**, 979 (1970).
14. Chemicals were added at the beginning of culture and left in the medium until the glands were harvested 6 or 8 hours later. The chemicals listed gave significant ($P < .05$) results in comparison with control cultures and are expressed as the ratio or percentage of control values for each separate experiment.
15. The values of NAT prior to culture were 2.72 nmole per pineal gland per hour for control glands from chickens killed in the light; placing the glands in organ culture resulted in initial culture control NAT of 2.66 nmole per pineal gland per hour—not a significant difference. The value of NAT prior to culture of control glands from chickens killed in the dark was 25.69 nmole per pineal gland per hour (9.4 times the light time value); placing the dark time glands in culture reduced the NAT to 10.35 nmole per pineal gland per hour, a loss of 60 percent of the activity. We know from previous work [S. Binkley, J. Riebman, K. Reilly, *Comp. Biochem. Physiol.*, in press] that dark time NAT is unstable and attribute the loss going into culture to that instability. The values for the controls are averages for the 11 "light-killed" experiments (44 control animals) and the 4 "dark-killed" experiments (16 control animals).
16. F. Stephan and I. Zucker, *Proc. Natl. Acad. Sci. U.S.A.* **59**, 1583 (1972); M. H. Stetson and M. Watson-Whitmyre, *Science* **191**, 197 (1976).
17. We thank J. Stephens. Support was provided to S.A.B. by NSF PCM 76-09682 and PCM 77-26474, Temple grant-in-aids, and a Temple research incentive award.

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# r and K Selection in Experimental Populations of *Escherichia coli*

Abstract. *Populations were adapted in vitro under density-dependent and density-independent population controls. Comparison of strains fails to demonstrate any trade-off in adaptation under these population controls.*

Although early efforts in ecology and population dynamics have been concerned principally with identifying and describing the action of population controls, more recent studies have sought to relate those controls to particular species' life history features. The most prominent such theory terms the action of population controls on species characters as r or K selection.

The term r selection refers to selection exerted by a density-independent type of population control under which growth is continuous and the population infrequently or never reaches its limit (*1*). This is thought to selectively favor an increased growth rate, a reduced body size, and reduced interspecific competitive ability. The term K selection refers to the effects of density-dependent regulation where the population is usually at its saturation density and seldom undergoes prolonged periods of growth. Natural selection here promotes a reduced growth rate, increased body size, and greater efficiency in competition and the use of resources.

Although this hypothesis makes numerous explicit predictions regarding the effects of these types of selection on particular life history features, the most fundamental provision of this theory is that adaptation under r and K selection results in a trade-off. That is, the effects of selection are opposed: Adaptation to K selection reduces fitness or effectiveness under the conditions in which r selection occurs and vice versa.

Since unpredictable weather is thought to function as a density-independent control, the effects of r and K selection are assumed to be continuously distributed along climatic gradients, with K selection (density-dependence) predominating in stable climates and r selection (density-independence) predominating where climate is harsh and unpredictable. Tests of this hypothesis, therefore, have often consisted of extensive comparisons of life history features in populations distributed along climatic or latitudinal gradients (*2*).

To determine whether populations are controlled dependently or independently of density is difficult, however, at best (*3*). Such studies, therefore, usually fail to attribute the observed demographic features with surety to either type of control. Nevertheless, the terms r selected or K selected are often applied to species simply because their life history features fit the expected pattern (*4*).

I report here a direct experimental test of this theory. In this study, r and K selection are applied to experimental populations of prokaryotic organisms in controlled environments, and adaptations in life history features under these controls

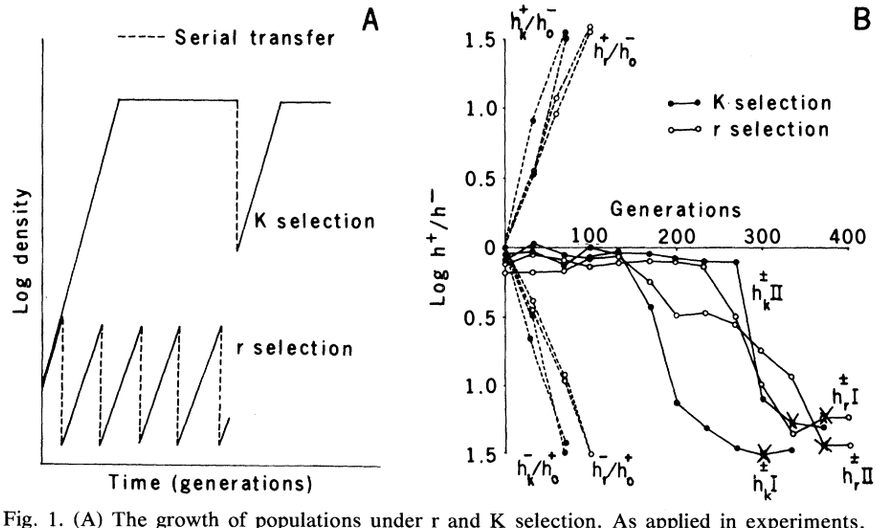


Fig. 1. (A) The growth of populations under r and K selection. As applied in experiments, populations under density-independent control are rarefied by serial transfer before reaching high density. Under density-dependent control, populations are usually at their limit. (B) The ratio of markers ($h^+/h^-$) diverges as strains adapt under r and K selection. Crosses (×) indicate the point at which adapted strains were isolated and repurified from populations under selection. Rapidly diverging ratios of $h^+/h^-$ (dashed lines) show the superiority of adapted strains ($h^{\pm}_K$ or $h^{\pm}_r$) in competition with parental strains ($h^+_0$).


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