(nonpolar) side chains (*8*). These side chains would then react with appropriate reagents to produce reactive (polar) functions. It was shown that the combination of polyglycine with formaldehyde yields seryl residues and with acetaldehyde yields threonyl residues (*8*). Thus, the presence and potential interference of the hydroxyl group during the process of formation of peptide bonds is overcome.

Apparently little has been done to elucidate means by which other reactive residues could have originated without interference with polymerization to α-peptides. To demonstrate that a peptide bond could easily form with the γ-carboxyl group of glutamic acid, the following experiment was performed. An aqueous solution was prepared containing 0.01*M* glycine (^{14}C-labeled), 0.01*M* L-cysteine, 0.01*M* L-glutamic acid, 0.1*N* HCl (final concentrations) and 2 mg of bentonite, a clay mineral that increases the yield of synthetic peptide (*9*). To the mixture, which was stirred with a magnetic stirrer, successive portions of sodium dicyanamide solution were added at 2-minute intervals, so as to bring the total concentration of the condensing agent to 0.12*M*. The solution was then neutralized with NaOH, and the clay was removed by centrifugation. A portion of the supernatant was chromatographed on Whatman No. 1 paper, with a standard of reduced glutathione (γ-glutamyl-cysteinyl-glycine) run in parallel, the solvent used being a mixture of isopropanol, formic acid, and water (65 : 1 : 34, by volume). The standard was located by spraying with *o*-tolidine (*10*), and a radioactive product of similar R_F, as observed by autoradiography, was eluted. Standard glutathione was added to the eluate which was then spotted on another sheet of Whatman No. 1 paper. Chromatography was carried out in the first dimension with a mixture of *n*-butanol, acetic acid, and water (25 : 6 : 25, by volume) as solvent. The second dimension was resolved by electrophoresis in borate buffer at *p*H 9.2, with 5.8 volt/cm applied voltage. The tolidine spray indicated that the shape and position of the carrier glutathione was the same as that of one of the labeled products that had been observed with x-ray film. Thus, under conditions where the γ-peptide bond may not be desirable, as usual with peptide synthesis, its production could pose problems. Similarly, in the pyrocondensation of aspartic acid, at least 33 percent of the peptide linkages formed were β-linkages (*11*).

The aforesaid means for the production of reactive side chains could have contributed, at least in part, to the formation of compounds needed for the ultimate appearance of living organisms. The overall scheme is based primarily on the production of polymers of amino acid residues with nonpolar side chains, such as alanine and glycine. This event could have taken place by any of a number of demonstrated experimental methods. These units, by interaction with nitrogen, water, and carbon dioxide, are convertible to residues with carboxyl and amino functions in their side chains. As part of a previously formed peptide chain, these residues, now transformed to species with reactive side chains, would not have interfered with the original polymerization process. Such a system is simple enough to suggest that it could have contributed to prebiological chemical evolution.

The study of the synthesis of α,β-diaminopropionic acid is not altogether out of place in the context of prebiological events since α,γ-diaminobutyric acid and α,δ-diaminovaleric acid have been found in bacterial peptides (*12*). It is conceivable that lysine (α,ϵ-diaminocaproic acid) is the product of a line of evolution that originally began with α,β-diaminopropionic acid.

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21 October 1966

Activity Rhythms and Adiurnal Light-Dark Control

Abstract. *The running-wheel activity pattern of mature male rats was successfully synchronized to light-dark cycles as long as 48 hours and as short as 16 hours. Even after 6 months' exposure to "days" longer than the normal 24 hours, the animals returned promptly to circadian rhythmicity when placed under free-running conditions of continuous dark. That such rhythms also reappeared when the light condition of the 36-hour cycle was reduced from 660 to 33 lumens per square meter suggests that brightness may be the critical factor in the unexpectedly broad range of entrainment demonstrated.*

Many circadian (near-24-hour) rhythms in living organisms "entrain" (that is, modify their spontaneous frequencies so as to synchronize) with imposed environmental cycles, particularly of light and dark (LD) (*1*). In parallel to natural conditions, most observations of physiological and behavioral entrainment have been made on diurnal (precisely 24-hour) schedules. In order to contribute to the understanding of the intrinsic timing mechanism, and particularly to explore the possibility of modifying its control, attempts have been made to establish entrainment to adiurnal (non-24-hour) LD cycles. As an appropriate and convenient dependent variable, the activity pattern of the rodent has been the choice of a number of investigators. The limited success of these efforts, against the background of other plant and animal experiments, has suggested the proposition that the more complex the organism, the greater the resistance to entrainment to period lengths deviating considerably from 24 hours (*2, 3*). Bruce (*3*, p. 38), for example, found it impossible to synchronize the hamster's wheel-running activity to "days" shorter than 23 or longer than 25 hours. In mice, even when departures from 24 hours were made in 1-hour steps, with extended habituation permitted at each stage, Tribukait (*4*) was unable to stretch the limits of entrainability beyond 21 and 27 hours (spring-cage activity). While the directly observed

activity of Bolles and Ogilvie's (*5*) rats remained synchronized with LD cycles as they were gradually lengthened to 28 hours, the cyclicity was virtually abolished during a week at 29 hours (*6*).

We have demonstrated far greater flexibility of activity pattern in the mature male albino rat (*7*). Wahmann (LC-34) activity cages were modified to permit continuous housing in the revolving drum, with such supplies of food and water that the animals needed to be disturbed no more than once a week. Continuous white noise of 75 ± 3 decibels masked extraneous auditory cues, partitions between wheels eliminated visual stimulation between animals, and room temperature and humidity were maintained close to 24.5°C and 50 percent. Illumination was by overhead fluorescent fixtures; except during the last phase of the experiment, the brightness at the wheels was 660 ± 110 lu/m^2 during the light periods.

After 10 days in the wheels, on a normal schedule of 12-hour light and 12-hour dark (LD 12:12), our first six animals were subjected to progressively lengthened "days." Extensive experience was given on the first three steps: 35 days of 26-hour duration (LD 13:13), 30 days of LD 13:14, and 23 days of LD 14:14. Inasmuch as the activity of four of the subjects seemed to have remained closely under the control of the light changes, the pace of the investigation was accelerated, with 1 hour added to the cycle every 4 to 9 days, until the 36-hour day was reached. These individuals continued to maintain the predominantly darktime running typical of the rat, as is illustrated in the Esterline Angus record of subject E's 7½ days on LD 18:18 (Fig. 1). Print-out counter scores for each of this cycle's 36 hours, averaged across cycles, give scant indication that the limit of entrainability had been reached for this animal (Fig. 2).

The next procedure was to ascertain whether exposure for 6 calendar months to adiurnal cycles had abolished, or distorted appreciably, the spontaneous periodicity of approximately 24 hours. When continuous darkness was imposed for 144 hours, the normal circadian rhythmicity appeared in the running patterns of all six animals.

Having established entrainment to a 36-hour cycle by the method of small

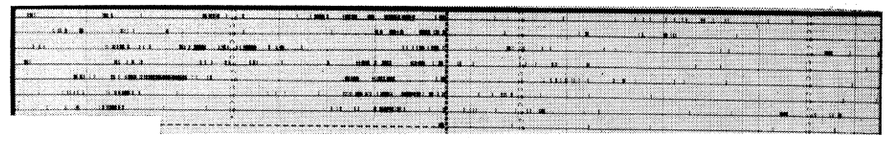

Fig. 1. Activity of subject E during 7½ consecutive cycles of 18-hour light and 18-hour dark. Each line represents a complete 36-hour cycle, with the first "day" at the top. Time reads from light onset at the right margin, through the dashed vertical line in the middle denoting dark onset, to the termination of dark at the left margin. Esterline Angus recorder speed was 0.19 cm/hour.

increments, it seemed reasonable to inquire whether synchronization might also be effected by sudden presentation of lengthened day. Therefore the continuous dark regimen was followed immediately by a return to the LD 18:18 schedule for six more cycles. Again, the same four animals entrained, almost as clearly as before. In addition, one of six naive rats which had entered the experiment during the 144 hours of continuous dark gave a comparable response. To push the limit of entrainability further, a shift was then made directly to LD 24:24. While it is true that on this schedule the activity during the last few hours of each dark period tended to be low, for the five entraining rats there was no intrusion of a prominent activity peak during the light, such as might signal a return to circadian rhythmicity. Finally, after 96 hours in continuous darkness, an abrupt reduction to cycles of LD 8:8 was made, with moderately successful synchronization of ten animals.

Why did we fail to find limits of entrainability as narrow as previous work had led us to expect? Species and apparatus differences could not immediately be ruled out. But an even more likely possibility was disparity in illumination, since Tribukait's observations were made at 66 lu/m^2 (6 foot candles) and ours at 660 (*8*). Our final procedure therefore was to observe the running of the 12 subjects on the LD 18:18 schedule, first with brightness level of 660 lu/m^2 during the light half of each cycle (eight 36-hour "days"), and then with a reduction to 33 ± 5.5 lu/m^2 (seven 36-hour days). The results were unequivocal: for even our best subject there was sudden failure to follow control by illumination at the lowered intensity. Instead, there could readily be identified for nine of the rats the emergence of an unmistakable circadian rhythm, as if they had been returned to continuous darkness (Fig. 3).

The conclusion seems inescapable that, given sufficiently high intensity of

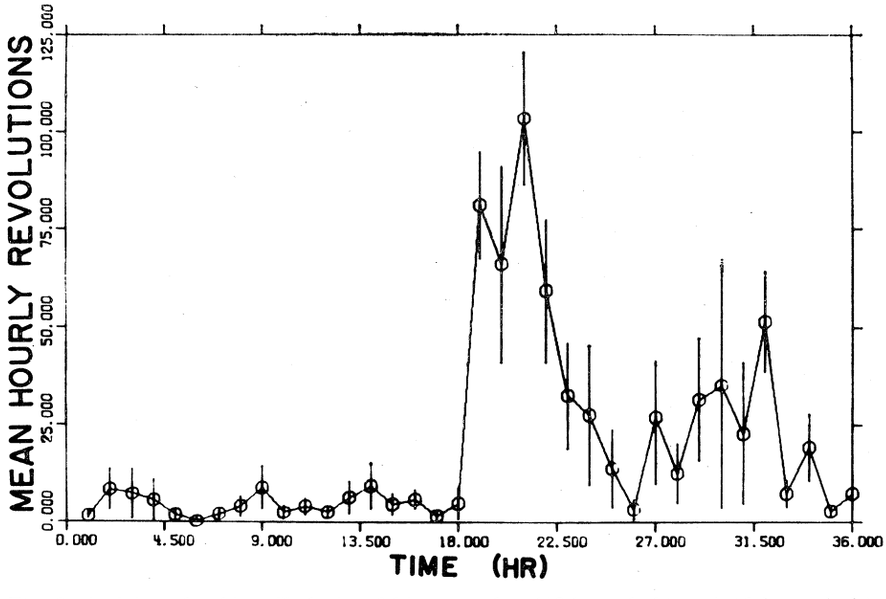

Fig. 2. Activity of subject E during 36-hour cycles (18-hour light and 18-hour dark). Each point represents number of wheel revolutions during that hour, averaged across seven consecutive cycles; vertical lines indicate standard error of the mean. Time reads from left to right, with dark onset at 18.000 hours.

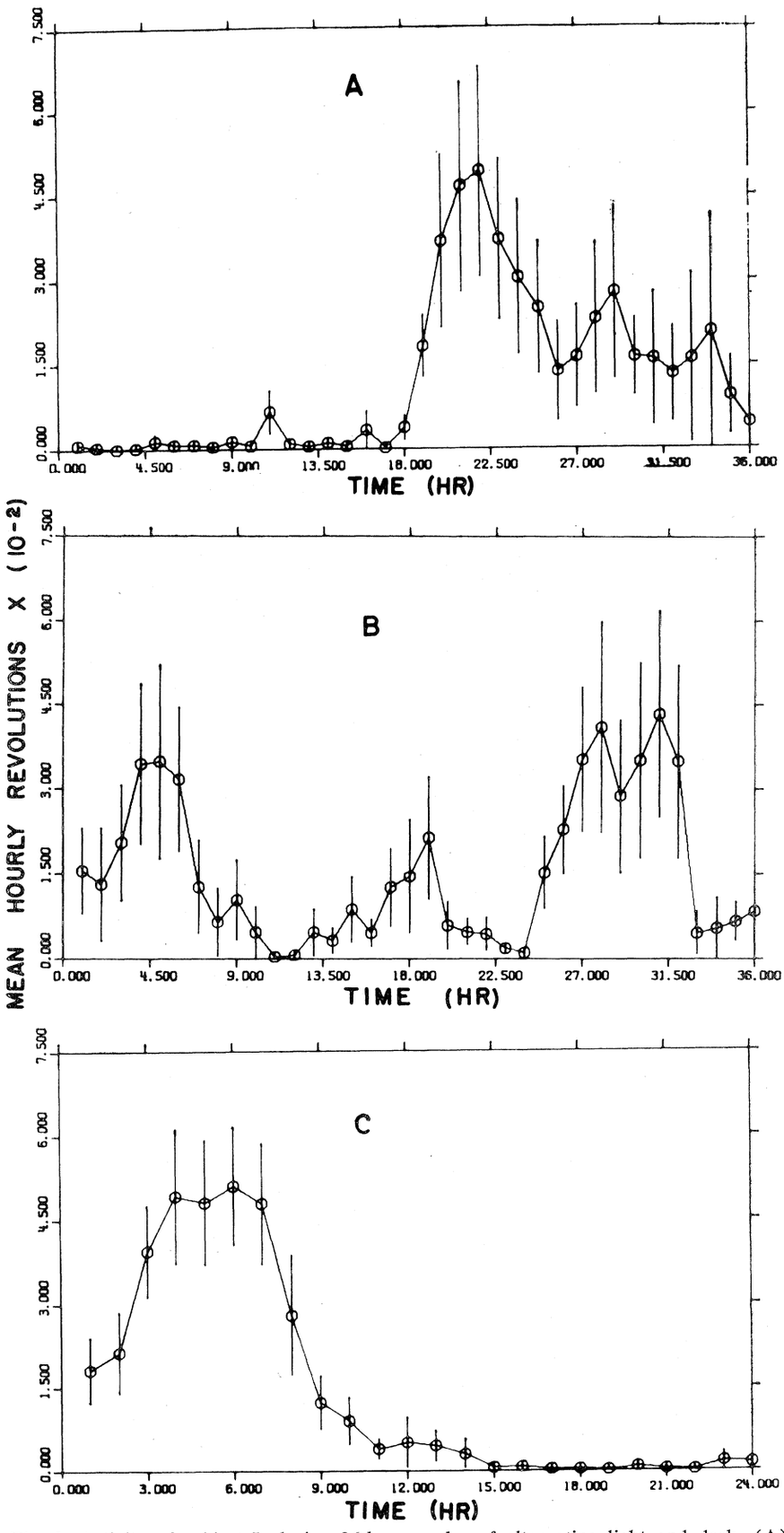

Fig. 3. Activity of subject L during 36-hour cycles of alternating light and dark. (A) Illumination during hours 0–18.000 is 660 ± 110 lu/m². (B) Illumination during hours 0–18.000 is 33 ± 5.5 lu/m². In A and B each point represents number of wheel revolutions for the given hour, averaged across the several cycles. (C) Data of B, re-averaged on a 24-hour scale, without regard for light-dark schedule.

illumination, the wheel-running of rats can be forced into a cycle period deviating from 24 hours more strikingly than has previously been reported at this phyletic level. There is evidence that, for at least some individuals, experience with cycles of intermediate length is not prerequisite for the development of such synchronization. The question arises whether this behavioral control reflects a corresponding modification of an internal "clock." It might be, instead, that the direct effect of illumination on activity overrode any influence by the clock, which was continuing on its own natural circadian period, in a way independent of illumination and related in only random fashion to the behavior recorded. That such uncoupling of activity from the clock did not occur is suggested by the observation that the first burst of running after shift to continuous darkness typically followed the onset of running during the just-preceding 36- or 48-hour day by very nearly 24 hours (*9*). The regularity of this phase relationship is consistent with the interpretation that the adiurnal LD schedule had established control not only over the general activity but also over the intrinsic timing mechanism itself.

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7. A more complete account of methodology and

results is given in M. L. Goff, thesis, Univ. of Virginia, 1965.

8. The effect of variation in intensity has been examined principally under conditions of continuous illumination. According to a recent statement of the "circadian rule," "With increasing intensities of illumination, the circadian period is shortened in diurnal (light-active) animals and lengthened in nocturnal (dark-active) animals." J. Aschoff, *Science* **148**, 1427 (1965). Intensity of illumination has been shown to influence also the phase relation between activity and synchronizing LD cycles of 24-hour or near-24-hour period: J. T. Enright, in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), p. 112. Some of our entrained animals exhibited regular phase lead, mostly of 2 to 4 hours, but only with LD cycles of less than 30-hour period.
9. There were seven instances of clear synchronization persisting to the end of such LD 18:18 or LD 24:24 treatment. The intervals (to the nearest hour) between the onset of activity during the last adiurnal cycle and successive activity onsets in continuous darkness were, respectively, 24, 24, 25, 25, 23, 24, 25 (Subject C after 18:18); 26, 23, 23 (Subject C after 24 : 24); 25, 20, 25, 26, 26, 24 (Subject E after 18:18); 36, 22, 21 (Subject E after 24:24); 24, 23, 22, 23, 24, 23, 24 (Subject F after 18:18); 25, 23, 25, 23 (Subject F after 24:24); 25, 23, 24, 23 (Subject L after 24:24).
10. Supported in part by PHS grant MH 04920. We acknowledge the discussions with our colleagues in the Psychology Department and with F. M. Ferguson and M. E. Perkins of the Computer Science Center.

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Adrenalectomy and Coat Color in Deer Mice

Abstract. *Adrenalectomy in prairie deer mice is followed by a profound darkening of the fur which occurs within 1 to 3 months. The phenomenon is most noticeable on the normally unpigmented ventral surfaces which turn dark gray or black. A possible mechanism for such hyperpigmentation would involve increased release of melanocyte-stimulating hormones.*

Partial dependence of coat color on endocrines is known for a variety of species. Examples are the well-documented effects of some steroids on avian breeding plumage and the effect of melanocyte-stimulating hormones on skin pigmentation in amphibia. Of a much rarer nature is evidence relating color to endocrine function in mammals. In this report we document a relationship between adrenal insufficiency and coat color in prairie deer mice *Peromyscus maniculatus bairdii*.

Colony-born deer mice (12 of each sex) were adrenalectomized at 9 to 10 weeks of age. Postoperative maintenance included 1 percent saline as a drinking solution and a single injection of 80 μg of cortisone acetate (intraperitoneally, in saline) immediately after the operation. An additional six deer mice of each sex (same age) were sham-operated, and another six were left as intact controls. Sham operations consisted of entry into the abdominal cavity only, with no postoperative injections of hormone. Normal coloration of adult *bairdii* is described by Hamilton (*1*) as: fur of the upper parts, brownish-gray mixed with darker hairs; fur of the under parts, gray at their bases with unpigmented tips, giving an all white appearance; and the above two patterns extended to the tail, making it sharply bicolored (hairs completely unpigmented on the ventral surface of the tail). Coat color in the present experiment was graded on an arbitrary 0 to 4 scale with class 0 equivalent to the normal animal just described. Classes 1 to 4 were separated as follows: (i) normal, but with bicolored feature of the tail less distinct and belly slightly more gray; (ii) ventral surfaces, including the tail, definitely gray; (iii) dark-gray ventral surfaces, tail evenly colored top and bottom, normally brown dorsal surfaces noticeably darker (often in blotches); and (iv) animal approaching black on all surfaces, with the exception of the hairs on the ankle region and on the male's scrotum. Classes 0, 2, and 4 are illustrated in Fig. 1.

Darkening of fur became noticeable about 1 month after removal of the adrenals and increased in magnitude for the next 1 to 2 months when a large degree of stabilization occurred. Table 1 shows frequency distributions of the various categories of animals 3 to 6 months after operations. No differences were apparent between intact and sham-operated controls. Adrenalectomy resulted in an obvious but somewhat variable darkening of the coat color (independent of sex). The majority of the adrenalectomized animals turned various shades of gray, a factor most obvious on the normally unpigmented belly and underside of the tail. Two animals turned almost coal black, and two darkened only slightly. No skin pigmentation changes were detected under gross examination. The completeness of adrenalectomy was confirmed in all animals included in Table 1.

Table 1. Frequency distribution of coat color classes of adrenalectomized or control deer mice 3 to 6 months after operations. Sham-operated and intact control groups were pooled.

Treatment	Sex	No.	Color classes				
			0	1	2	3	4
Controls	♂	12	10	2	0	0	0
	♀	12	11	1	0	0	0
Adrenalectomized	♂	8	0	0	4	4	0
	♀	12	0	2	4	4	2

Several possible mechanisms could be postulated to explain the observed changes in hair pigmentation. The most

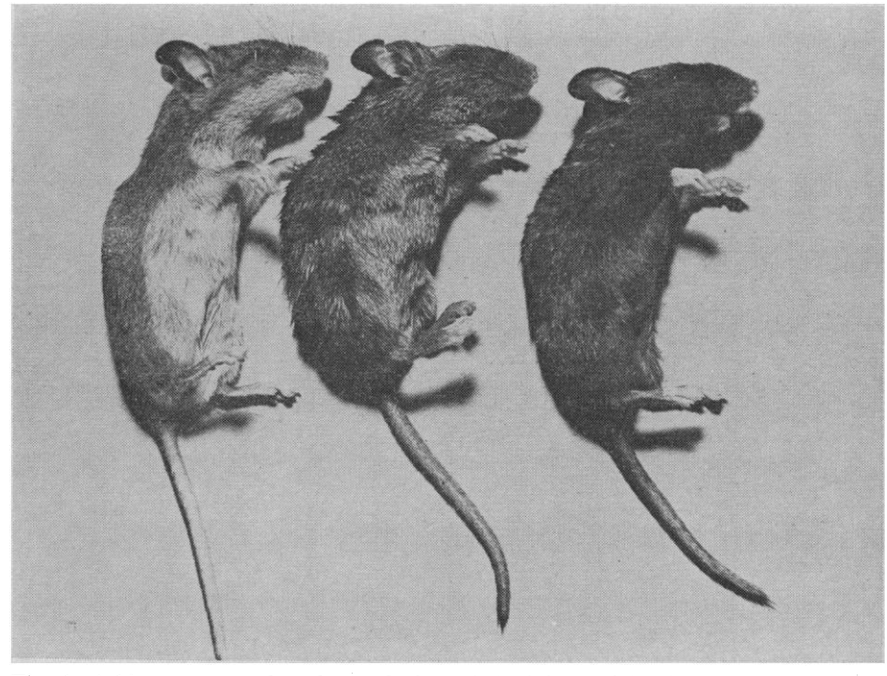

Fig. 1. Arbitrary coat color classes 0, 2, and 4 (left to right) in female deer mice. Class 0 represents a sham-operated control; classes 2 and 4 are adrenalectomized.