hypothalamus are commonly seen after staining with PTA; thus, all agranular vesicles seen after osmium preparation may not be chemically identical. The dense-core vesicles have been associated with storage sites of certain biogenic amines (*10*). However, staining with PTA is not dependent upon the reduction of a metallic oxidant as are the histochemical reactions used to demonstrate catechols and indoles (*11*). Therefore, the staining of vesicles with PTA would not necessarily reflect their content of biogenic amines. The staining of vesicle cores with PTA could indicate that a matrix material of similar chemical reactivity is shared by certain agranular vesicles and also possibly by the dense-core synaptic vesicles. Although no correlation between the PTA-stained vesicle cores and the PTA-stained presynaptic tufts or projections (*4*) has been demonstrated, their possession of similar chemical affinity for PTA suggests a possible relationship.

The dense material associated with specialized intercellular junctions (*12*) in nonneuronal tissue also stains selectively with alcoholic PTA in glutaraldehyde-fixed tissue. In junctions of the macula adherens type (*12*) between neighboring ependymal cells and adjacent adrenal medullary cells, there is an electron-opaque material resembling the electron-opaque band subjacent to the postsynaptic membrane in both form and staining intensity. At the nonneuronal intercellular junctions, the material stained by PTA is bilaterally symmetrical, as if in mirror images. Thus, only at synaptic sites is the stained material arranged in a polarized, oriented fashion; this fact suggests that the function, if not the composition, of the nonneuronal specialized contact zones is different (*1, 4*).

Phosphotungstic acid has a high molecular weight and has been used as a precipitating agent in the isolation of basic amino acids (*13*). The staining of collagen by PTA has been related to its binding to specific basic amino acids (*14*). The special material at synapses may be a protein containing a high proportion of basic amino acids such as lysine, histidine, and arginine. Basic proteins are also found associated with nuclear DNA (*15*) and may account for the affinity of the nuclear material for PTA. To analyze which broad classes of cellular macromolecular components might be causative in the alcoholic-PTA staining of synpatic material, we treated 80-μ frozen sections of glutaraldehyde-fixed paraventricular hypothalamus with deoxyribonuclease, ribonuclease (*16*), pepsin, trypsin, and testicular hyaluronidase. Of these treatments, only proteolysis removed the PTA-stained material. The synaptic vesicles and membranes of tissue stained with osmium after proteolytic digestion appeared well preserved. Thus, the proteolytic digestions selectively remove the material stained at synapses by alcoholic PTA without disturbing membranous components. Although this type of cytochemical experimentation cannot be taken as definitively identifying the PTA-reactive synaptic material, the involvement of protein is strongly suggested.

Our results indicate that synaptic sites have, in addition to their membranes, a component distinguished by its affinity for PTA. This material, which may be proteinaceous, is in a special spatial orientation with respect to interneuronal transmission. The electron-microscopic staining procedure which reveals this material to be distinct from synaptic membranes offers an additional approach to the cytochemical analysis of the synapse. The further characterization of this material as to structural and enzymatic composition, functional turnover, and chemical binding properties may clarify its significance in the formation and function of synapses.

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Open-Field Behavior in Mice: Evidence for a Major Gene Effect Mediated by the Visual System

Abstract. *In segregating F_2, F_3, and F_4 generations, albino mice had lower activity and higher defecation scores than pigmented animals when tested in a brightly lighted open field. These differences persisted when members of an F_5 generation were tested under white light, but largely disappeared under red light. Thus it was concluded that there is a major gene effect on the quantitative traits of open-field activity and defecation which is mediated by the visual system and that albino mice are more photophobic than pigmented mice under conditions of bright illumination.*

Merrell (*1*) has recently reviewed studies reporting the effects of single genes on behavior and has suggested that the study of gene substitutions, one or a few at a time, may provide information not only about the genetics of a quantitative behavioral trait, but about the trait itself. In a recent quantitative

Table 1. Mean transformed open-field activity and defecation scores of albino and pigmented mice.

Generation	Activity*			Defecation†			N	
	Albino	Pigmented	Difference‡	Albino	Pigmented	Difference‡	Albino	Pigmented
F_2	10.65	12.67	2.02±.35	1.97	1.76	0.21±.069	152	485
F_3	10.04	12.34	2.30±.37	2.24	1.81	.43±.062	180	661
F_4	10.14	12.47	2.33±.51	2.28	1.84	.44±.077	113	315

* Mean activity scores were obtained from transformed data, where each subject's score is the square root of the total activity over the 2-day test. † Mean defecation scores were obtained from transformed data, where x = (total boluses + ½)½. ‡ Absolute difference ± standard error of the difference between the means. All differences were highly significant ($p < .001$), with the exception of that for defecation in the F_2 generation, which was significant at the 0.01 level of probability.

genetic analysis of open-field behavior in mice (*2*), evidence for a possible major gene effect was obtained. The present report examines this effect in more detail.

Two inbred strains of mice (BALB/cJ and C57BL/6J) which differ widely in open field behavior were crossed to produce an F_1 generation; F_2 and F_3 generations were then obtained by random matings. At 40 ± 5 days of age, each animal was tested for 3 minutes on each of two successive days in a brightly lighted open field (36 by 36 inches) (90 by 90 cm). The floor and sides (8 inches high) of the field were of white, painted Plexiglas. Two sets of five light sources were beamed through holes and red filters to photoconductive cells on the opposite side, effectively dividing the floor into 36 squares, 6 by 6 inches each. The number of light beams broken was automatically recorded on counters. Two 20-watt fluorescent tubes mounted 37 inches above the floor of the field provided the only illumination during testing. The total number of light beams interrupted and the total number of fecal boluses dropped during the two test periods were used as activity and defecation scores. Due to heterogeneous group variances in the raw data, both activity and defecation scores were subjected to square root transformations. In addition, since defecation scores were low, 0.5 was added to these raw scores prior to transformation.

Inbred C57BL/6 mice are non-agouti black (*aaBBCC*), whereas BALB/c mice are albino (*AAbbcc*). In the F_2 and subsequent generations, five coat colors occur: agouti, cinnamon, black, brown, and albino (*3*). Although open-field activity and defecation are clearly influenced by genes at many loci (*2*), it is of interest to compare mean scores when the data are grouped according to coat color. The mean activity and defecation scores among the four pigmented classes in the F_2 and F_3 generations did not differ, but pigmented animals were consistently more active and defecated less than albinos (see Table 1). Animals of the F_4 generation which were included in a selection experiment were also examined. The mean behavioral scores of albino and pigmented mice in each of six lines were obtained and unweighted means of these means are also presented in Table 1.

The BALB/c strain shows a pattern of relatively low activity and high defecation in the open field when compared to the C57BL/6 strain. The transformed mean activity scores of the BALB/c and C57BL/6 strains were 4.46 and 16.06, respectively, while those for defecation were 2.88 and 1.07 (*2*). Therefore, the difference between the behavioral scores of albino and pigmented animals in the F_2 and later generations may be due in part to the effects of closely linked loci rather than entirely to the *c*-locus itself. If this is actually the case, and if strain differences exist at these closely linked loci, other results might have been obtained had this study been conducted with mice from other foundation stocks.

From the present results, however, it was concluded that the observed differences in open-field behavior between albino and pigmented animals in the F_2 and later generations were due at least in part to the effect of a single gene at the *c*-locus. Since animals in the four pigmented classes have pigmented eyes and albinos have nonpigmented eyes, it was hypothesized that this single gene effect is mediated through the visual system and that albinos are more photophobic than pigmented animals in the brightly lighted test situation. A second experiment was performed to test this hypothesis.

McClearn (*4*) has demonstrated that the difference in open-field activity between a low-active inbred albino strain (A/Crgl) and a high-active inbred black strain (C57BL/Crgl) of mice decreased somewhat under red light. Under such illumination, visual stimulation should be low or absent for mice. Therefore, if the differences we observed were due to a greater photophobic reaction of albinos, these differences should be decreased or eliminated when tested under conditions of red illumination. Animals from an F_4 control population were randomly mated to produce an F_5 generation which was tested under two conditions of illumination. White light was provided as before, while red light was provided by a 60-watt, ruby-red incandescent bulb. Pigmented animals were taken only from litters in which albinos were tested and light conditions were assigned at random, resulting in a 2^3 factorial arrangement of treatments (illumination, pigmentation, and sex). Subclass numbers ranged from 18 to 20 and a least squares analysis of variance employing unequal subclass numbers was used (*5*).

Analysis of activity scores indicated that main effects due to pigmentation ($F = 14.13$, $p < .001$, df = 1, 144), illumination ($F = 18.65$, $p < .001$), and their interaction ($F = 6.34$, $p < 0.2$) were significant. The mean transformed activity scores of albino and pigmented animals under white light were 8.80 and 12.91, respectively, whereas those for albino and pigmented animals under red light were 13.28 and 14.10. Post hoc comparisons among these four means indicated that albinos tested under white light were significantly ($p < .001$) less active than each of the other three groups, which did not differ among themselves. Therefore, both main effects and their interaction were primarily due to the large effect of the white light condition upon albinos. This finding is consistent with the hypothesis that albinos are more photophobic than pigmented animals when tested in the open field under the usual conditions of bright illumination. The main effect and interactions involving sex did not approach significance.

The mean transformed defecation scores of albino and pigmented animals under white light were 2.10 and 1.95, respectively, while those of albino and pigmented animals under red light were 1.73 and 1.76. Since activity and defecation are negatively correlated (*2, 6*), the pattern of these means corresponds closely to that which would have been predicted from the

activity data. However, only the main effect due to illumination was significant ($F = 5.92$, $p < .02$).

The results of this study provide evidence for a major gene effect on the quantitative traits of open-field activity and defecation. Analyses of variance of the behavioral scores of albino and pigmented animals in the F_2, F_3, and F_4 generations indicate that this single gene difference at the *c*-locus may account for approximately 12 percent of the variance in both traits. As compared to pigmented animals, albinos show a pattern of higher defecation and lower activity when tested under white light. This behavioral pattern, which is interpreted as indicating heightened emotionality (*6*), largely disappears when animals are tested under red illumination; thus, the differences observed under bright illumination appear to be the result of a fear reaction. Therefore, it is concluded that this single gene effect is mediated through the visual system and that albinos are more photophobic than pigmented animals.

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Entrainment of Circadian Rhythms by Sound in Passer domesticus

Abstract. *The circadian locomotor rhythm of house sparrows was entrained by a sound stimulus. The birds were maintained at a constant temperature in dim green light. The entraining agent was 4½ hours of tape-recorded bird song played each day. Variations in the response to this stimulus have been correlated with individual variations in free-running period. This is the first clear demonstration that a biological clock can be influenced by sound stimuli.*

Light and temperature cycles of various durations and magnitudes can entrain the circadian rhythms of many organisms (*1, 2*). But the effectiveness of an agent other than light or temperature has not been adequately demonstrated, although several reports indicate such a possibility (*3*). Enright (*2*) observed that a loud buzzer served as a weak entraining agent for the activity rhythm of one house finch. Data of Halberg *et al.* (*4*) suggest that blind mice may have been entrained by normal mice that were entrained to a light cycle in the same room. Human rhythms can be entrained by social and psychological factors, but these factors are at present poorly defined (*5*). Attempts to find other agents for various organisms have failed (*6*). There are no published reports in which circadian rhythms in any organism have been rigorously shown to entrain to any clearly defined environmental factor other than light and temperature.

In order to demonstrate that a signal is an effective entraining agent, one must force the rhythm examined to assume the same frequency as that of the signal. In addition, to exclude the possibility that the rhythm is masked (that is, the measured parameter is forced but not the underlying timing system) it is necessary to show that the phase of the rhythm has been shifted during entrainment. The free-running period of the organism must always be examined under constant conditions in order to verify phase shifting by the entraining signal and to rule out coincidence between the free-running period of the organism and the period of the signal.

Our experience with house sparrows (*Passer domesticus*) housed in individual light-tight but not sound-proof boxes suggested that bird song might act to entrain the circadian rhythm of locomotor activity and led us to investigate this possibility systematically. The stimulus consisted of tape recordings of the sounds made by a colony of sparrows in the field in February as they began their activity in the early morning and as they went to roost in the evening (*7*). The sound was played continuously for approximately 4½ hours per a 24-hour day and was repeated each day at the same time. Its intensity was between 70 and 80 db; the background sound in the incubator was 60 to 70 db.

Ten sparrows (five males and five females) were examined for ability to be entrained by this stimulus. The responses did not vary according to sex (Table 1). Three birds produced activity records which satisfy all the rigorous criteria for entrainment. All three had circadian free-running periods preceding entrainment. During entrainment (26, 30, and 38 days), the stimulus controlled both the phase and the period of the rhythms. When the sound was discontinued, all three ran once more with a circadian period, the phase of which indicated that the rhythm had been phase shifted by the entraining signal (Fig. 1A).

Two other birds showed phase and period control by the sound stimulus until it was discontinued, but they failed to satisfy rigorous criteria of entrainment. In one case the subsequent free-run was indistinguishable from 24 hours, and in the other the sound was discontinued too soon to adequately demonstrate that it had shifted the phase of the rhythm.

Three other birds showed good phase and period control by the sound but subsequently broke away from the entraining signal and assumed circadian periods in its presence (Fig. 1B). In these three cases the sound caused a shift in the phase of the rhythm. In the remaining two cases the sound stimulus had no influence on the phase or period of the locomotor rhythm.

In each case the effect of the sound stimulus can be clearly related to the free-running period of the bird. Table 1 summarizes the period lengths and entrainment behavior of all ten birds. Note that in all situations in which entrainment failed (the subsequent periods of birds 69, 5, and 74, as well as the periods preceding entrainment of birds 4 and 7) the length of the period falls outside the range 23 hours 35 minutes to 24 hours 45 minutes. All other free-running periods fall within this range. The particular stimulus used in these experiments (presented with a period of 24 hours) apparently will entrain only those birds whose free-running periods fall within these limits.

With the exception of birds 4 and 7, there were differences in free-running period between the beginning and the