icilloyl-ε-aminocaproic acid (Table 1).

Thus, incubation in vitro with BSA of spleen cell suspensions derived from rabbits immunized with DNP-BSA led to a definite stimulation of the formation of antibodies with specificity directed toward the dinitrophenyl hapten (Table 1 and Fig. 1). The titer of antibodies toward the dinitrophenyl group in cultures challenged with BSA was similar to that obtained with DNP-BSA. The specificity of the antibodies was corroborated by the specific inhibition of modified phage inactivation (Table 2). Moreover, we have some results which show that the anamnestic immune response toward the hapten of cultures stimulated with BSA could be detected also by a modification of the Jerne technique (*11*) in which erythrocytes coated with the dinitrophenyl hapten were used.

Our results agree with those of Dixon and Maurer (*12*), who found an increase of antibodies to human serum albumin upon subsequent immunization with chicken γ-globulin, but not with those of Ovary and Benacerraf (*13*) who reported a lack of effect in vivo of carrier protein injected secondarily on production of antibodies against the hapten used for primary immunization.

The stimulation in vitro with DNP-lysozyme of the formation of antibodies against the dinitrophenyl group by cells derived from animals immunized both with DNP-BSA and with lysozyme confirms previous findings (*1, 3, 4*) and is in agreement with the hypothesis of cell-to-cell interaction. Our observation on the stimulation with the protein carrier alone may be related to the "original antigenic sin" (*14*). The boosting effect of BSA would be, in this case, due to protein areas which share complete determinants with the dinitrophenyl hapten (*15*). On the other hand, the finding reported may mean that an initial stage in the antigen recognition by a cell may involve the intact immunogenic macromolecule, and thus allow an array of different specificities to be stimulated. The recognition at the level of an intact immunogen has been demonstrated also in connection with studies on the role of conformation and net electrical charge in antigenicity (*16*).

ANDREI SULICA
REBECA TARRAB
MICHAEL SELA
*Department of Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel*

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## Photosensitivity of the Circadian Rhythm and of Visual Receptors in Carotenoid-Depleted Drosophila

Abstract. Drosophila melanogaster *was raised on aseptic diets, with and without β-carotene. The sensitivity of visual receptors in the carotenoid-depleted flies was lowered 3 log units, but the photosensitivity of the circadian rhythm was not affected. This result suggests that the chromophore of the photopigment which mediates light effects on the circadian rhythm is not a carotenoid derivative.*

Circadian rhythms are affected by light in a variety of ways. They can be entrained to cycles of light intensity, initiated and phase-shifted by single light pulses, inhibited by constant bright light, and changed in free-running period by changes in light intensity (*1*). What is the location and nature of the photopigments that mediate these effects?

The precise location of the photopigment is not known for any organism, but evidence is accumulating that extraoptic sites are involved. Light impinging directly on the brain can entrain circadian rhythms in amphibians (*2*), reptiles (*3*), and birds (*4*); and an extraoptic site—probably the brain—is also implicated in insects (*5, 6*). However, there is evidence that in mammals the eyes might be the photoreceptor (*7*).

The nature of the photopigments that mediate light effects on circadian rhythms is also unknown. Action spectra have been determined for light effects on circadian rhythms in plants (*8*), protozoans (*9*), and insects (*10, 11*), but this approach has not greatly narrowed the choice of compounds.

Another method of determining the nature of the photopigment of insect circadian rhythms is reported here. It takes advantage of the fact that, in all of the chemically known visual systems, the chromophore of the photopigment (rhodopsin) is the carotenoid derivative retinaldehyde (vitamin A aldehyde). Carotenoids are synthesized only by plants, and carotenoid deficiency in insect diets results in impairment and loss of visual sensitivity (*12, 13*). If the chromophore of the photopigment of the circadian rhythm is a carotenoid derivative, we might expect the circadian rhythm of carotenoid-depleted insects to be less sensitive to light than normal. Here we report experiments in which *Drosophila melanogaster* was raised on aseptic diets with and without β-carotene and then tested for both photosensitivity of the compound eye and photosensitivity of the circadian rhythm of adult emergence.

The aseptic diet used was similar to that devised by Sang (*14*), except that β-carotene was added in some cases. The diet (125 ml) was placed in polypropylene flasks and autoclaved; sterile fructose was then added to all of the flasks, and β-carotene, dissolved in ethyl alcohol (0.02 mg/ml), was added to some of the flasks.

*Drosophila* eggs were collected and sterilized (*14*). They were rinsed with sterile distilled water and added (in

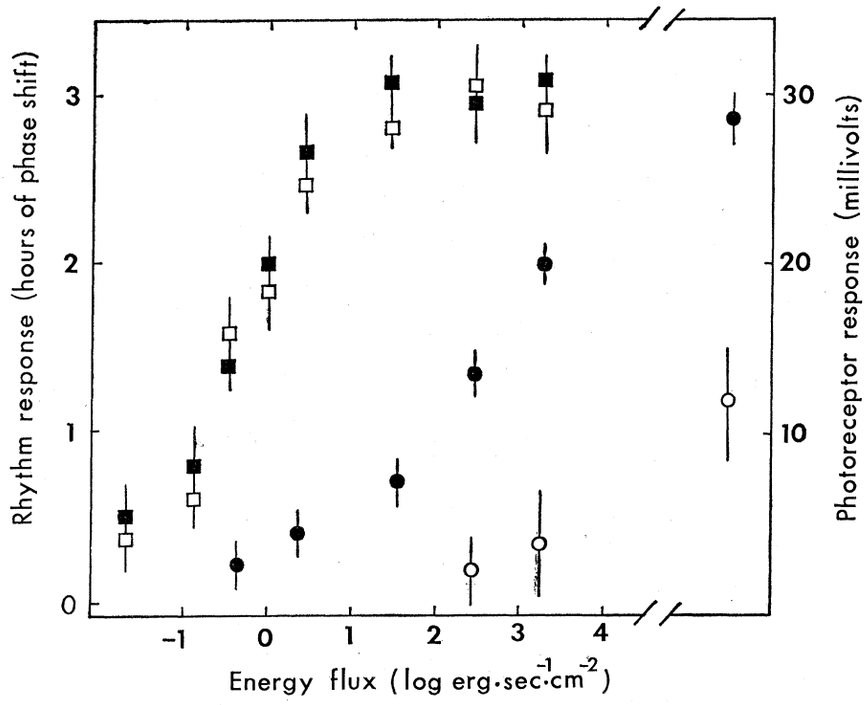


Fig. 1. Response-energy curves for phase shifts of the circadian rhythm, and for the receptor component of the retinal action potential of *Drosophila melanogaster* grown on aseptic diets with β-carotene (closed symbols) and without β-carotene (open symbols). Squares indicate the phase delay in hours (left ordinate) of the circadian rhythm resulting from 15-minute monochromatic light pulses (458 or 454 nm). Circles indicate the height in millivolts (right ordinate) of the sustained corneal negative wave of the retinal action potential elicited by monochromatic light flashes (454 nm) of approximately 1 second. The electrophysiological recordings (circles) were carried out on some of the same flies used in the circadian rhythm experiment (squares). The energy scale gives the light flux used in both experiments, but the exposures were 900 times longer for resetting the rhythm. The responses of the visual receptors shown to the right of the break in the abscissa were obtained with a bright white light. Vertical lines through symbols indicate standard errors of the mean (phase shifts of rhythm: four to six replicate experients; electrophysiological recordings: eight to ten carotenoid-supplemented flies; four to five carotenoid-depleted flies).

suspension) to the flasks containing the artificial diet. The flasks were then placed in incubators at 25°C and maintined with a light:dark cycle of 12 hours light and 12 hours dark.

Fifteen days later the pupae were harvested by flotation and placed on a flat, black surface in light- and temperature-controlled fraction collectors (*11, 15*), which provide an hourly record of adult emergence. The mortality from egg to adult was about 40 percent, and attempts to grow *Drosophila* more than one generation on the diet did not succeed.

The assay of the photosensitivity of the circadian rhythm was the amount of phase delay of the peak of adult emergence generated by 15-minute monochromatic light signals. As in previously reported experiments (*6, 11*), "experimental" populations of pupae were exposed to the light signals 3 hours after the final dusk and left thereafter in constant dark. "Free-run" control populations were not exposed to a light signal. The phase shift generated by the light signals—median emergence hour of the experimental population minus that of the free-run control—was measured 2 days after the light signal. Each experiment at a given intensity was repeated four to six times, using interference filters with a transmission peak at 454 or 458 nm.

The assay of the photosensitivity of visual receptors was the height, in millivolts, of the sustained corneal negative wave elicited in the dark-adapted eye by monochromatic light flashes of approximately 1 second; this sustained negative wave is caused by the electrical response of the photoreceptor (retinular) cells to light (*16*). The techniques used to record the retinal action potentials of insects have been described (*6, 17*). All the flies for the electrophysiological measurements came from the same populations as those used for the experiments on the circadian rhythm, and the same interference filter was used (454 nm).

Figure 1 shows the relation between the intensity of the stimulus and the responses of the circadian rhythm and of the visual receptors in flies grown aseptically, with and without β-carotene. The photosensitivity of the visual receptors in carotenoid-depleted flies is about 3 log units lower than that of carotenoid-supplemented flies. This result is similar to that reported for the housefly, where one generation of carotenoid deprivation lowered visual sensitivity by 2 log units (*12, 13*).

There is no significant difference, however, between the carotenoid-depleted and carotenoid-supplemented flies with regard to photosensitivity of the circadian rhythm; the standard errors of the means of these measurements overlap.

The fact that carotenoid deprivation impairs visual sensitivity but does not impair the photosensitivity of the circadian rhythm suggests that a carotenoid-derived chromophore is not involved in mediating light effects on the circadian rhythm in *Drosophila.* Another indirect reason for thinking that a different photopigment may be involved is the finding that the spectral sensitivity curves of insect circadian rhythms are quite similar to each other, but they differ from the spectral sensitivity curves of compound eyes (*6*) as well as from the absorption spectra of common carotenoids (*18*) .

Alternatively, it could be argued that the photopigment of the circadian rhythm does have a carotenoid-derived chromophore, but that one generation of carotenoid deprivation is not sufficient to lower the level of this photopigment. In insects there is good evidence for carotenoid transmission through the egg (*19*), and circadian rhythms are present and light sensitive in early larval and embryonic stages (*6, 20*); it is thus conceivable that in *Drosophila* some carotenoids are transmitted through the egg and preferentially utilized at an early stage in the formation of a photopigment for the circadian rhythm. Although this interpretation seems considerably less likely to be correct than the first one, it is not excluded by the results.

WILLIAM F. ZIMMERMAN
*Department of Biology, Amherst College, Amherst, Massachusetts 01002*
TIMOTHY H. GOLDSMITH
*Department of Biology, Yale University, New Haven, Connecticut 06520*

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## Narcotic Tolerance and Dependence: Lack of Relationship with Serotonin Turnover in the Brain


Abstract. *Serotonin turnover was measured in mouse brain by means of the conversion of radioactivity from labeled tryptophan into serotonin. Animals with a high degree of tolerance to and physical dependence on morphine did not differ from control mice.*


Recent studies have suggested that serotonin pathways in the central nervous system might be involved in the mediation of the opiate effect, even though brain serotonin concentrations do not change during the development of tolerance and physical dependence (*1*). Way and his co-workers (*2*) showed that, after treatment with pargyline, there is a significant increase of brain serotonin in morphine-tolerant mice, which they interpreted as an indication of increased serotonin turnover. *p*-Chlorophenylalanine (PCPA), which inhibits brain tryptophan hydroxylase (*3*), was found to antagonize the acute analgesic effect of morphine in rats (*4*) and to inhibit the development of tolerance and physical dependence in mice (*2*).

In the experiments described here tolerance and physical dependence were produced in mice by the implantation and retention for 3.7 days of a specially prepared 75-mg morphine pellet (*5*). Control mice were subjected to the same operative procedure without pellet implantation. Tolerance to opiate-induced running activity was demonstrated by placing ten implanted mice and ten control mice in cumulative counter cages for 1 hour, then injecting them with levorphanol (20 mg/kg). The running activity (*6*), measured for 30 minutes after the injection, was found to be $11.9 \pm 2.6$ beam crossings per minute in the control mice and $0.05 \pm 0.03$ beam crossing per minute in the implanted mice. We determined whether mice were physically dependent on morphine by using the narcotic antagonist, naloxone, to precipitate the jumping syndrome described by Maggiolo and Huidobro (*7*). The degree of dependence was quantitated from the number of mice jumping off a platform within 10 minutes of the naloxone injection, as described by Way *et al.* (*8*). Control mice would not jump at any dose of naloxone up to the $ED_{50}$ (median effective dose) for convulsions ($150 \pm 13$ mg/kg). The $ED_{50}$ of naloxone for jumping in the implanted mice at 3.7 days was $0.75 \pm 0.25$ mg/kg.

We measured serotonin turnover in male Swiss-Webster mice (National Institutes of Health, Bethesda, Md.) (24 to 26 g) by a direct method employ-

Table 1. Conversion of [$^3$H]tryptophan to serotonin in brains of tolerant-dependent mice (3.7 days after implantation with 75-mg morphine pellets) and in control (sham-implanted) mice; L-[$^3$H]tryptophan (1 c/mmole, 500 $\mu$c/kg) was injected intravenously at zero time. Four mice were used in each group. Data are the means ± the standard errors of the means; dpm, disintegrations per minute. See text for criteria of tolerance and dependence.

| Treatment | Minutes after injection | Tryptophan content of brain (nmole/g) | Brain tryptophan specific radioactivity (dpm/nmole) | Serotonin content of brain (nmole/g) | Brain serotonin specific radioactivity (dpm/nmole) | Conversion index* (nmole $g^{-1}$ $min^{-1}$) |
|---|---|---|---|---|---|---|
| Control | 10 | 22.0 ± 0.8 | 6322 ± 207 | 2.80 ± 0.27 | 3365 ± 102 | 0.12 ± 0.007 |
| Tolerant-dependent | 10 | 20.1 ± 2.2 | 7434 ± 613 | 2.50 ± 0.25 | 4344 ± 335 | 0.12 ± 0.005 |
| Control | 20 | 21.1 ± 1.6 | 2412 ± 247 | 3.15 ± 0.13 | 2305 ± 372 | 0.12 ± 0.05 |
| Tolerant-dependent | 20 | 21.2 ± 1.6 | 2742 ± 143 | 3.08 ± 0.27 | 2827 ± 114 | 0.14 ± 0.025 |
| Control | 40 | 17.7 ± 3.2 | 764 ± 109 | 2.55 ± 0.25 | 1613 ± 220 | 0.12 ± 0.03 |
| Tolerant-dependent | 40 | 23.4 ± 1.0 | 1285 ± 82 | 3.15 ± 0.26 | 2526 ± 200 | 0.13 ± 0.01 |
| Control | 80 | 16.3 ± 1.8 | 361 ± 28 | 2.78 ± 0.31 | 1014 ± 34 | 0.083 ± 0.013 |
| Tolerant-dependent | 80 | 18.3 ± 1.6 | 438 ± 41 | 2.85 ± 0.06 | 1231 ± 461 | 0.10 ± 0.01 |
| Control | 160 | 18.6 ± 1.2 | 286 ± 32 | 2.58 ± 0.15 | 937 ± 88 | 0.046 ± 0.007 |
| Tolerant-dependent | 160 | 21.9 ± 2.2 | 313 ± 15 | 2.88 ± 0.27 | 744 ± 24 | 0.04 ± 0.003 |

* From Eq. 1 in text.