pupae received 15-minute monochromatic light signals simultaneously with the signal given to the white light control population. The phase shift caused by the monochromatic signal was expressed as a percentage of the phase shift caused by the white light signal. In successive experiments, the intensity of the monochromatic light signals was altered until the phase shift 7 days after the signal equaled approximately 50 percent (Fig. 2). The spectral regions tested extended from 367 nm into the infrared (*10*).

Differences in wavelength did not affect the direction of phase shift—that is, whether the phase was advanced or delayed. The magnitude of the phase shift increased with increased light intensity, but reached a maximum that was equivalent to the phase shift caused by the intense white light control signal. Finally, during the 7-day period after the light signals, phase shifts caused by dim light signals changed relative to phase shifts caused by intense signals, such as the white light control signal (Fig. 1).

The action spectrum for delay phase shifts is similar to the action spectrum for advance phase shifts (Fig. 2). In both cases, the most effective wavelengths were between 420 and 480 nm; there was a slightly reduced response to the shorter wavelengths, and a sharp cutoff above 500 nm. Wavelengths of 570 nm and longer induced neither advance nor delay phase shifts, even at very high intensities. Phase shifts could not be induced by intense, nonmonochromatic light spanning a broad spectral region from 600 nm into the infrared (*10*). Finally, the two curves are superimposable within the limits of experimental resolution (Fig. 2).

Interpretation of the action spectra is difficult because of lack of knowledge concerning the precise photoreceptive site within the insect and the light-transmitting effects of interfering pigments. However, the *Drosophila* action spectra are similar to action spectra for regulation of circadian rhythms in the fungus *Neurospora* and the moth *Pectinophora* (*2, 5*). The insensitivity of the *Drosophila, Neurospora,* and *Pectinophora* rhythms to wavelengths above 570 nm is noteworthy, because action spectra for light effects on circadian rhythms in other organisms show a small but distinct peak between 600 and 700 nm after a sharp decrease near 450 nm (*3, 11*). Insensitivity above 600 nm is evidence against the possible involvement of phytochrome, a photoreceptive pigment associated with photoperiodism in plants (*12*).

The similarity of the action spectra for advance and delay phase shifts strongly supports the view that the same kind of photoreceptive pigment mediates the two shifts. The action spectra are consistent with the possibility that a carotenoid is the photoreceptor, but they do not rule out pterins, flavins, or other compounds.

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Cycasin: Detection of Associated Mutagenic Activity in vivo

Abstract. *Cycasin and its lagycone, methylazoxymethanol, increase the mutant frequency of* Salmonella typhimurium *histidine auxotrophs when tested in the host-mediated assay. As expected, the degree of cycasin-related mutagenic activity depends on the facility with which the compound can be enzymatically deglucosylated by the normal intestinal flora.*

Plants in the family Cycadaceae, genus *Cycas*, contain a β-glucoside, cycasin, which is toxic (*1*) and carcinogenic (*2*) when administered orally to mammals. Comparative studies with gnotobiotic and conventional rats have shown that cycasin must be enzymatically converted to its aglycone, methylazoxymethanol, by the normal intestinal flora before death or tumors can be induced (*3, 4*).

Methylazoxymethanol, in contrast to cycasin, is mutagenic for *Salmonella typhimurium* (*5*) and causes chromosomal aberrations in onion root-tip cells (*6*), but little is known about the formation or retention of mutagenic activity in vivo.

The recently introduced "host-mediated" assay (*7*), which incorporates a microbial indicator in a murine host, presents an ideal system for studying this problem. Histidine auxotrophs of *Salmonella typhimurium* are injected intraperitoneally, and the test compound is administered directly (orally, intramuscularly, and so forth) to the host. Thus the compound potentially

Table 1. Effect of a reduced intestinal bacteria population on the mutagenic capacity of cycasin in the host-mediated assay. Intestinal flora and *Salmonella* were counted as viable organisms per milliliter. *Salmonella* were recovered from the peritoneal cavity.

Group	Treatment		Viable organisms (No./ml)		
	Ampicillin	Cycasin	Intestinal flora	*Salmonella*	Mutant frequency
Positive control	−	+	1.86×10^7	6.91×10^7	4.9×10^{-7}
Ampicillin control	+	−	5.79×10^4	1.51×10^8	3.3×10^{-8}
Negative control	−	−	5.38×10^6	6.89×10^7	2.9×10^{-8}
Experimental	+	+	3.46×10^4	1.29×10^8	5.4×10^{-8}

can be metabolized, and possibly activated, by the animal before the indicator organisms are encountered. After a 2-hour incubation period, the *Salmonella* are removed aseptically from the peritoneum and plated on mimimum and complete media to determine the ratio of mutants to wild type. The resulting mutant frequency (MF) is compared to that occurring spontaneously, and thus can serve as an indicator of relative mutagenicity. We used this method to detect mutagenic activity after the oral administration of cycasin and cycasin aglycone.

To detect and enumerate reversions from histidine dependence (auxotrophs) to histidine independence (prototrophs) in *S. typhimurium* G46, we used a modification of a previously described method (*7*). Male Swiss albino mice (25 to 30 g) were usually treated in groups of four. One-half milliliter of either 2 percent cycasin or 1 percent methylazoxymethanol was administered by oral intubation to each animal. A 4-hour static culture of *Salmonella* in tryptone broth was diluted with saline (1:4); after the appropriate time interval, the mice were injected intraperitoneally with 2.0 ml of this dilution. Two hours after the injection, samples of peritoneal fluid were collected and pooled before titrating.

Host-mediated studies indicated that the oral administration of 2 percent cycasin or 1 percent methylazoxymethanol would result in an increased MF for *S. typhimurium* G46. When the compounds were given to the mice 2 hours before the organisms were introduced, the MF was increased 30-fold by cycasin and 100-fold by the aglycone (the spontaneous MF was approximately 1×10^{-8}).

The mutagenic activity of cycasin, if due to its conversion to the aglycone, should depend on the time allowed for activation, the route of administration, and the presence of a normal gut flora to bring about the necessary hydrolysis. As the time between the administration of the compound and the injection of the indicator organisms is increased, more of the cycasin is metabolized to the active state. The mutant frequency in a control group treated with cycasin (2 percent) was 1.1×10^{-8}; that in the group treated with the aglycone (1 percent) was 1.6×10^{-8}. As the time between administration of the drug and the organism was increased from 0 to 2 hours, the MF in the cycasin-treated animals increased linearly, whereas that

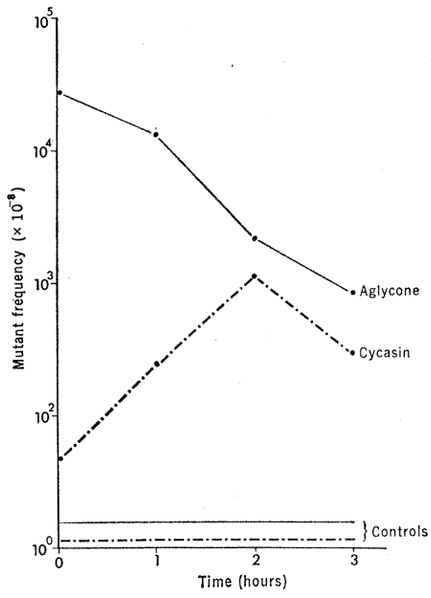

Fig. 1. Effect of time [hours between treating the mice with cycasin or its aglycone methylazoxymethanol and the indicator microorganisms] on the mutant frequency. Mutant frequency is the ratio of prototrophs to auxotrophs among the *Salmonella* after 2 hours in the host.

in mice treated with the aglycone decreased (Fig. 1). The latter decrease continued for as long as 3 hours (the longest period tested). The MF for organisms in the cycasin-treated mice also began to decrease between 2 and 3 hours. After 2 hours, most of the cycasin had evidently been hydrolyzed. The optimum time, therefore, for administering 2 percent cycasin orally is 2 hours before the *Salmonella* are introduced.

By using various means of administration, we obtained further evidence that the increase in MF for organisms in cycasin-treated mice was due to the activation by intestinal bacteria. Mice were treated intraperitoneally, orally, intravenously, or intramuscularly. Each group received one dose of cycasin (0.5 ml of a solution containing 20 mg/ml, orally or intraperitoneally; 0.1 ml of a solution containing 50 mg/ml, intramuscularly or intravenously) 2 hours before the organisms were injected; controls received saline. As expected, cycasin given parenterally did not cause an increase over the spontaneous MF. In the mice which received cycasin orally, the MF was increased from the control level of 4×10^{-9} to 2×10^{-6}. This is in agreement with an earlier report that cycasin is inactive if given to adult animals by injection (*4*).

A significant reduction in the numbers of intestinal bacteria which hydrolyze cycasin should diminish the mutagenic activity inasmuch as mammalian tissue does not have sufficient β-glucosidase (*3, 8*). Ampicillin (Bristol Laboratories) was used to partially sterilize the large intestine. Mice were treated with (i) cycasin (10 mg orally) 2 hours before injection of *Salmonella*; (ii) ampicillin (2 mg orally) 20 hours and 4 hours before injection of *Salmonella*; (iii) neither compound; or (iv) both compounds. Intestinal bacteria were enumerated by use of the cecum contents as an indicator of the population. After the peritoneal fluid was withdrawn from four mice, the ceca were removed, rinsed in sterile saline to remove adhering *Salmonella*, and minced in 10.0 ml of saline. Samples were then titrated by standard bacteriological procedures; intestinal contents, prototrophs, and auxotrophs were plated in triplicate.

A comparison of the intestinal and peritoneal cell counts reveals that the ampicillin treatment reduced the intestinal cell counts approximately 100-fold without affecting the *Salmonella* titer (Table 1). Cycasin alone (positive control) resulted in the anticipated rise in MF for *S. typhimurium*; ampicillin alone did not alter the spontaneous MF (negative control).

The MF in the mice treated with both ampicillin and cycasin was approximately the same as that of the negative controls, probably because the mice treated with the antibiotic to lower the intestinal bacterial population could not convert cycasin to its aglycone. This essential role of the indigenous microorganisms is in keeping with previous reports that germfree rats, in contrast to conventional animals, excrete cycasin quantitatively (*8*) and fail to develop tumors after ingesting cycasin (*9*).

These results indicate that cycasin-related mutagenic activity, as manifested in increased frequency of reversion to histidine independence for *S. typhimurium* G46, can be detected by the host-mediated assay. Production of this activity is facilitated by an oral route of administration, a normal gut flora in the host to metabolically convert the compound, and enough time for the necessary enzymatic conversion to occur.

These findings are especially pertinent because this is the first instance of mutagenic activity of cycasin introduced in an assay system. Since the

procedure required for the preparation of cycasin aglycone is complex (*4, 10*), this host-mediated method may be advantageous for further study of cycasin and related compounds with additional microbial indicators.

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Prostaglandin Stimulation of Rat Corticosteroidogenesis

Abstract. *Prostaglandins and their C20:ω6 fatty acid precursors are present in rat adrenal glands. Small doses of prostaglandins (PGE_1, PGE_2, or $PGF_{1\alpha}$, 1.4 to 2.4 micromolar) increased steroidogenesis in the superfused adrenal glands obtained from hypophysectomized rats. This effect was mimicked in part by both adrenocorticotropin and its postulated intracellular intermediate adenosine 3′,5′-cyclic monophosphate; all three responses were inhibited by cycloheximide.*

The suggestion that prostaglandins may regulate the intracellular concentration of adenosine 3′,5′-cyclic monophosphate (cyclic AMP), the postulated intracellular intermediate of hormonal action (*1*), was made at the Nobel Symposium on prostaglandins (*2*) and this hypothesis was based on the finding that prostaglandin efflux from adipose tissue was directly associated with hormonal stimulated lipolysis (*3*), a mechanism known to involve cyclic AMP and which can be inhibited by PGE_1 (*4*); PGE_1 was known to inhibit other hormonal responses, namely, the permeability response of toad bladder (*5*) and kidney tubules to vasopressin (*6*).

In adipose tissue a hormone-activated lipase was believed to be involved in prostaglandin release; this released fatty acids including bis-homo-γ-linolenic acid, and this C20:ω6-polyenoic acid underwent cyclization and oxidation by the microsomal prostaglandin synthetase to yield PGE_1 (*7*); PGE_1 then acted back to prevent further formation of cyclic AMP, thus serving as a feedback regulator of the lipolytic mechanism. This hypothesis was subsequently extended to the rat gastric mucosa where the hormonal response (acid secretion) was also believed to be mediated via cyclic AMP. Prostaglandin and free fatty acid release from the mucosa was detected, and reapplication of PGE_1 resulted in inhibition of the acid secretion induced by nervous, drug, or hormonal stimulation (*8*).

As in most tissues, prostaglandins are present in the rat adrenal gland (*9*), but unexpectedly the concentrations of prostaglandins present and released from the gland were reduced on adrenocorticotropin (ACTH) stimulation of the adrenal. However, more prostaglandins may be obtained from the superfusate than can be directly extracted from the gland. Furthermore, there are relatively large concentrations of the C20:ω6 precursor of PGE_2 and $PGR_{2\alpha}$ present in the form of cholesterol arachidonate (*10*).

In this report we now present data on the effect of prostaglandins on rat corticosteroidogenesis when a continuous superfusion method is used (*11*).

Female Sprague-Dawley rats (120 to 140 g) were obtained 3 to 6 hours after acute hypophysectomy by the parapharyngeal route (Charles River Breeding Laboratories, Inc.). The adrenal glands were removed, trimmed of fat, bisected, and decapsulated. Glands from not less than ten rats were used for each incubation; approximately 20 minutes elapsed from the time of killing to the beginning of the incubation during which time the glands were kept in ice-cold Krebs-Ringer bicarbonate solution. The bisects were superfused, in a volume of 2 ml at 0.4 ml/min, with Krebs-Ringer bicarbonate solution plus glucose (200 mg/100 ml). The medium was gassed with a mixture of oxygen and carbon dioxide (95:5), and the vessel was agitated in a Dubnoff shaker at 37°C. The effluent was collected every 30 minutes in an automatic fraction collector. One experiment consisted of two incubations (test and control), each lasting 5 to 6 hours. Corticosterone, the principal steroid secreted by the rat adrenal cortex, was assayed fluorimetrically (*12*) with an Aminco-Bowman fluorimeter. The contribution of nonspecific fluorescence when incubation media are assayed in vitro is almost negligible (*13*). Neither PGE_1, PGE_2, nor $PGF_{2\alpha}$ interfered with the corticosterone assay. Prostaglandins, cyclic AMP, ACTH, and cycloheximide were prepared in the superfusing medium.

During the first 1 to 2 hours a small but significant increased release of corticosterone was always observed (Fig. 1, open circles), which may be due to residual ACTH, since the phenomenon was not seen in rats that had been hypophysectomized 48 hours earlier. The rise may also be a consequence of the change from Krebs solution at 4°C to 37°C. The basal release of corticosterone was about 0.4 μg per rat per hour. The preparation responded to graded doses of ACTH and cyclic AMP with increased steroid output.

The compounds PGE_1 (2.8 μM), $PGF_{2\alpha}$ (2.8 μM), and PGE_2 (1.4 to 2.8 μM) increased steroidogenesis, PGE_2 being the most potent of these. Introduction of PGE_2 (2.8 μM) into the medium caused a rapid and highly significant increase in steroidogenesis which was maximal within 1 hour ($P < .01$). This stimulation was not sustained, and 3 hours after the start of the PGE_2 perfusion there was no significant difference in corticosterone secretion although the PGE_2 was in contact with the adrenals (Fig. 1).

The steroidogenic effect of PGE_2 was dose dependent. The maximum steroid response to 2.8 μM PGE_2 was 1.2 μg per rat per hour (Fig. 1) and that to 1.4 μM PGE_2 was 0.8 μg per rat per hour (Fig. 2). The lower limit for a detectable response was 0.28 μM. The response elicited by PGE_2, in contrast to that obtained by equipotent doses of ACTH and cyclic AMP, was transitory. The decay of the steroidogenic response to PGE_2 is clearly seen in Fig. 1 (2.8 μM) and in Fig. 2 (1.4 μM), where, in contrast to ACTH (0.5 milli-international unit per milliliter) and cyclic AMP (1.0 μM), PGE_2 did not exert a significant steroidogenic effect after 4 hours.

The steroidogenic response to graded