is too lengthy to pursue here, results from T experiments have been interpreted in terms of the external (*5*) or internal coincidence model (*6*).

Beck (*7*) concluded that induction of diapause in the European corn borer, *Ostrinia nubilalis*, is controlled by an hourglass that measures the dark phase. The optimal light-dark cycle for inducing diapause is 12 hours of light alternating with 12 hours of darkness (LD 12:12; T24). Pittendrigh (*8*) concurred, but in a reanalysis of Beck's data stressed that time measurement occurs most effectively when T = 24 hours, that is, when the circadian system is in what he termed resonance with the light cycle. When borers are raised with T's of other than 24 hours (for example, LD 24:12; T36) the amount of induction drops significantly (100 percent in T24; 65 percent in T36). A replotting of Beck's data by Pittendrigh yielded a circadian surface or topography with a peak at T24. Since Beck's experiments did not extend beyond T40 it was not possible to completely rule out circadian oscillations as the basis for photoperiodic time measurement in this species.

McLeod and Beck (*9*) have demonstrated that termination of diapause in *Ostrinia* is also photoperiodically controlled (optimal photoperiod, LD 16 : 8). Our T experiments show that the type of clock involved in this response resembles an hourglass. Larvae were reared from eggs in LD 12:12 at 30°C on an artificial diet (*10*). After about 22 days, the larvae reach the end of the last larval instar. They cease to feed and enter diapause. The incidence is invariably between 95 and 100 percent. Diapausing larvae were collected and placed in plastic containers (13 by 18 by 5 cm) layered with filter paper that was continuously saturated with distilled water. Twenty-five larvae were placed in each container, and all experiments consisted of four replicate containers. Within a week after the larvae had entered diapause, the containers were placed in lighttight black boxes containing ceiling-mounted 7-watt cool white fluorescent tubes (Sylvania F4T5/D), which were water-jacketed to prevent temperature cycles. The light intensity at floor level was 200 lux. The boxes were ventilated by a small fan connected to a system of baffles; they were kept in a temperature-controlled room at 30°C. Pupation was assayed daily as an indicator of diapause termination. We assume that termination is photoperiodically controlled and that postdiapause morphogenesis proceeds at a fixed rate which, as in *Drosophila* (*11*), is independent of photoperiod. Larval populations maintained at 30°C in LD 12:12 are characterized by high mortality (approximately 70 percent) and a mean time to pupation of about 50 days for the survivors, whereas populations switched to LD 16:8 have a mean pupation time of 14 days and low larval mortality (approximately 10 percent). By examining diapause termination rather than induction, the effects of long T's (for example, T60) can be studied without the added complications of larval feeding and the fact that developing larvae go through several complex stages in their rather brief (22 days at 30°C) developmental sequence before they enter diapause.

We first conducted a standard T experiment in which the light phase was held constant at 16 hours and was coupled with 8, 20, 32, or 44 hours of darkness (thus creating T's of 24, 36, 48, and 60 hours) (Fig. 1A). Neither the amount of light (constant in each T) nor periods of darkness greater than 8 hours are the measured intervals. Since a peak is not observed at T48, a circadian oscillation is probably not involved in time measurement.

The interpretations based on data derived from the standard T experiment were strengthened when we changed the experimental protocol. Instead of holding L constant, D was held constant at 8 hours, and T was varied by extending L. This experiment gave positive results in all cases (Fig. 1B). The interval that is measured and that results in termination of diapause is 8 hours of darkness. Photoperiodic time measurement occurs irrespective of the absolute value of T. The system thus acts like an hourglass, and the fact that the longer T's (for example, LD 52:8; T60) result in 100 percent pupation after 30 days indicates that the number of 8-hour dark phases required for maximum induction is small.

The only other clear case in insects where circadian oscillations have been ruled out in photoperiodic time measurement is in the aphid *Megoura viciae* (*12*). In this insect the production of sexual and parthenogenetic females is under photoperiodic control. Time measurement is effected by an hourglass mechanism.

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Tin: A Potent Inducer of Heme Oxygenase in Kidney

Abstract. *Tin greatly enhances heme breakdown in kidney, thus impairing heme-dependent cellular functions, such as cytochrome P-450 mediated drug biotransformation. This novel action of the metal results from a potent induction effect on heme oxygenase, the enzyme that catalyzes heme oxidation in microsomes. The possible toxicological implications of this tin effect in the kidney merit further investigation.*

Heme, derived from hemoglobin and various cellular hemoproteins, is degraded to biliverdin by the microsomal enzyme heme oxygenase (*1*). This enzyme is inducible in liver by certain endogenous compounds (*2*), as well as by certain trace and heavy metals (*3*). These metals are quite potent inducers of hepatic heme oxygenase, cadmium and cobalt, for example, enhancing the rate of heme oxidation from 5 to 15 times the normal rate. Metal induction of heme oxidation is associated with concomitant and marked inhibitory effects on heme-protein mediated cellular functions such as the cytochrome P-450 dependent detoxification of drugs, carcinogens, and environmental chemicals (*3*).

Normally the heme oxygenase activity of tissues other than liver and spleen is not great, nor have such tissues been shown to be particularly responsive to inducers of this enzyme activity. However, during the course of studies of

metal induction of this enzyme in liver and in certain nonhepatic tissues we have discovered that heme oxygenase in kidney is especially responsive to tin and that this trace metal induces the enzyme in kidney to a relative extent unmatched in our experience by metals in other tissues. This potent enzyme-inducing property of tin is a newly recognized biological action of the metal in the kidney and one that may have potential toxicological significance.

The following procedures were employed in these studies. Male Sprague-Dawley rats (150 to 200 g) were subcutaneously injected in the nuchal area once with a solution of stannous chloride at doses ranging from 2.5 to 25 μmole per 100 g of body weight (0.56 to 5.64 mg of $SnCl_2 \cdot 2H_2O$ per 100 g), or with other metals in doses as indicated. Sixteen hours after treatment, the animals were killed; homogenates and microsomal fractions were prepared from different organs and the following parameters of heme metabolism were examined: δ-aminolevulinate (ALA) synthetase, heme oxygenase, cytochrome P-450 dependent ethylmorphine demethylase, and microsomal cytochrome P-450 content (*4–8*).

It was found that in the liver, tin administered subcutaneously significantly altered some of these enzymic or other microsomal parameters (Table 1). This included a threefold increase in heme oxygenase activity and a 30 percent decrease in P-450 mediated drug metabolism and microsomal content of cytochrome P-450. The ALA-synthetase activity did not increase significantly. Intraperitoneal administration of the metal magnified the effects in liver by 15 to 20 percent. Tin did not have a pronounced inducing action on the heme-oxidizing activity of tissues such as the heart, the lungs, or the intestinal mucosa.

A very striking effect of the metal was, however, produced on the kidneys, with the rate of heme oxidation activity of this organ consistently between 20 and 30 times (Table 1, Fig. 1) that of control kidney 16 hours after a single treatment with stannous chloride. In one set of experiments (data not shown) heme oxidation activity exceeded 40 times the control level; intraperitoneal administration of the metal did not change the magnitude of the induction response. Concomitant with the striking increase in heme oxidation, the microsomal content of cytochrome P-450 in kidney was decreased by an average of 50 percent. The ALA-synthetase activity of the kidney was not altered in these short-term studies. The effect of tin on heme oxidation activity in the kidney was found to be dose-related (Fig. 1), with the metal, at a single dose as low as 2.5 μmole per 100 g, still being a potent inducer of this renal enzyme activity—this dose producing nearly a sixfold increase in the rate of heme breakdown. The inducing effect of tin on heme oxidation in kidney was not due to the presence of the metal in isolated microsomes since stannous chloride, when added (10 to 100 μ*M*) directly to the assay system, produced no change in the rate of heme degradation. Similarly, cobalt and other metals do not enhance this enzymic reaction when added to microsomes in vitro (*3*).

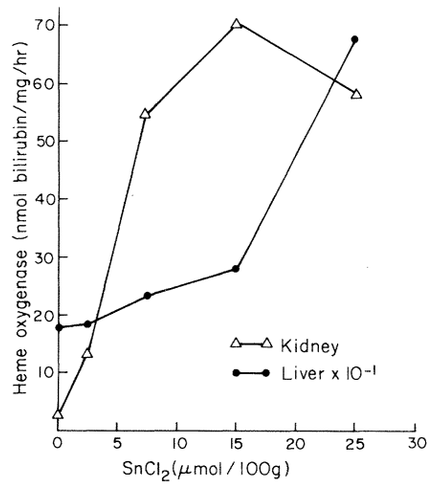

Fig. 1. Rats were treated with the doses of stannous chloride indicated above 16 hours before they were killed. Thereafter hepatic and kidney microsomal fractions were prepared and assayed for heme oxidation activity as described in the legend of Table 1.

Administration of tin daily for 3 days (dose, 25 μmole per 100 g) produced an approximately 35 percent decrease (data not shown) in contents of mitochondrial cytochromes a, b, c, and c_1 in both renal cortical and renal medullary tissue. One dose of tin did not significantly alter these mitochondrial cytochromes, as expected because of the substantially longer half-lives of these heme-proteins compared with the microsomal heme-protein cytochrome P-450.

The ability of tin to elicit an exaggerated induction of heme oxygenase in kidney was not matched by other trace elements (*9*), although all had to some extent heme-oxygenase inducing activity. All the metals tested were inhibitory for renal oxidative drug metabolizing activity, with this effect varying in degree. This inhibition is probably accounted for by the same mechanism through which such metals impair drug oxidation in liver (*3*)—that is, by a reduction of cellular content of cytochrome P-450, the terminal oxidase of the microsomal drug metabolizing enzyme complex.

Schwarz (*10*) has provided evidence that tin is an essential trace metal in rats, but the role of this metal in human physiology is unclear and difficult to study. The intake of tin mainly from foods appears to be highly variable and may range from very small amounts up to 40 mg/day (*11, 12*); the latter is five times

Table 1. Effect of stannous chloride on heme-related enzyme activities of kidney and liver. Rats were injected subcutaneously with stannous chloride (25 μmole per 100 g) and were starved; 16 hours later they were killed. The livers were perfused in situ with 0.9 percent NaCl and thereafter the livers and kidneys were homogenized in tris (hydroxymethyl)aminomethane hydrochloride buffer (0.5*M*, *p*H 7.4) containing 0.25*M* sucrose. Whole homogenates were used for the assay of ALA synthetase activity (expressed as nanomoles of ALA per milligram of protein per hour) (*4*). Microsomal fractions were prepared from both livers and kidneys as described previously (*5*) and various assays were carried out. Activities of hepatic and renal heme oxygenase were measured as described earlier (*6*) by incubating (10 minutes at 37°C) the microsomal fraction with an NADPH generating system, with hematin (17 μ*M*) serving as substrate. Heme oxygenase activity is expressed as nanomoles of bilirubin formed per milligram of protein per hour. Microsomal drug-metabolizing activity was determined as described earlier (*7*) by measuring oxidative demethylation of ethylmorphine; results are expressed as nanomoles of products (*p*-aminophenol) formed per milligram of protein per hour. The microsomal content of cytochrome P-450 was measured as described by Omura and Sato (*8*).

Organ	Activity (nmole mg^{-1} $hour^{-1}$)			Cytochrome P-450 (nmole mg^{-1})
	Heme oxygenase	Ethylmorphine *N*-demethylase	ALA synthetase	
Liver				
Control	2.3 (± 0.27)	191.0 (± 7.6)	44.1 (± 10.1)	0.75 (± 0.02)
Stannous chloride	6.8 (± 0.38)*	124.0 (± 18.6)*	46.0 (± 6.9)	0.55 (± 0.01)*
Kidney				
Control	2.5 (± 0.34)	70.5 (± 10.8)	75.8 (± 7.0)	0.07 (± 0.01)
Stannous chloride	58.0 (± 7.01)*	23.2 (± 8.4)*	86.6 (± 18.9)	0.04 (± 0.01)*

*$P < .05$.

higher on the basis of weight than the dose of tin that produced a sixfold increase of heme oxygenase activity in this study. Intestinal absorption of tin is low (*11*) but the body burden of the metal in humans is reported to equal that of cadmium and may substantially exceed that of trace metals such as cobalt, chromium, mercury, and nickel. As has been noted previously (*12*), however, the accuracy of quantitative data on inorganic tin contents of animal and human tissues, foods, and other domestic and commercial products is the subject of much doubt because of considerable loss of the metal during analytical procedures. Thus the extent of human exposure to tin is most likely greater than previously recognized.

The liver is, on a per unit basis, more active in cytochrome P-450 dependent oxidative metabolism of foreign chemicals than is the kidney (Table 1). However, the very potent inducing effect of tin on heme oxygenase in renal tissue is associated with a more exaggerated decrease in heme-dependent drug oxidation than is produced by the metal in liver. Since, as noted above, this tin-induced alteration in renal drug oxidative activity extends to other heme-dependent cellular functions as well, the potential toxicological consequences of the metal action described here merit further investigation.

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Theiler's Virus-Induced Demyelination: Prevention by Immunosuppression

Abstract. *The effect of immunosuppression with cyclophosphamide and rabbit antiserum to mouse thymocytes on demyelination induced by Theiler's virus in SJL/J mice was ascertained from Epon-embedded sections (1 micrometer) of the central nervous system. Immunosuppression not only eliminated mononuclear cell infiltrates in the spinal cord white matter, but it also prevented the occurrence of demyelination. These results suggest that demyelination in this infection is immune-mediated.*

It has been suggested that demyelination in multiple sclerosis may result from an immune-mediated response triggered by a virus infection of the central nervous system (CNS). However, evidence that a virus causes multiple sclerosis remains circumstantial (*1*). Further presumptive evidence for a viral etiology might come from the demonstration of a disease in animals like multiple sclerosis and produced by a virus. The essential criterion for such a model would be the selective pathological change of demyelination. Although there are several animal models of virus-induced demyelination that have been useful in studying the basic mechanisms of demyelination, in none has the destruction of myelin been demonstrated to be immunopathological (*2*).

Theiler's virus infection has been shown to produce primary demyelination in mice (*3*). Theiler's viruses are enteroviruses indigenous to colony-bred mice, and occasionally they may cause spontaneous paralytic disease (*4*). A study of the pathogenesis of Theiler's virus infection in 3-week-old, outbred Swiss mice has shown that limb paralysis developed 9 to 18 days after intracerebral inoculation of virus and was due to neuronal involvement (*3*). The distribution of the gray matter pathology was identical to that described for experimental poliomyelitis virus infection in monkeys (*5*). More importantly, all mice surviving CNS disease induced by Theiler's virus had persistent CNS infection, and between 1 and 6 months mononuclear cell infiltrates and demyelination were found in the spinal cord white matter (*3*). These pathologic findings have been substantiated by a temporal ultrastructural study (*6*). Although the early gray matter pathology appeared to be the result of direct cell lysis consistent with the known effects of enteroviruses in tissue culture, it seemed possible that the late developing white matter inflammation with concomitant demyelination might be immunopathological.

Preliminary immunosuppression experiments with outbred Swiss mice resulted in a uniformly high mortality before the effect of this treatment on demyelination could be evaluated (*7*). When 10- and 100-fold less virus was administered, demyelination did not regularly occur. Subsequently a number of inbred strains of mice were tested for susceptibility to Theiler's virus infection. The SJL/J strain proved a more suitable host than Swiss mice for studying the effect of immunosuppression on demyelination because (i) gray matter involvement is less severe (11 percent mortality compared to 48 percent), (ii) inflammation in the leptomeninges and white matter is more pronounced, and (iii) active demyelination occurs earlier—2 to 3 weeks after infection. By 2 months all surviving SJL/J mice develop a chronic neurologic disorder characterized by general inactivity and slowed movement, poor righting ability, and stimulus-sensitive extensor spasms of the limbs. In other respects, the pathogenesis of the infection in SJL/J mice is similar to that already described in Swiss mice.

In our experiments 3-week-old, male SJL/J mice (Jackson Laboratory) were inoculated intracerebrally with 1000 suckling mouse mean lethal doses ($SMLD_{50}$) of the DA strain of Theiler's virus. Infected mice received cyclophosphamide, rabbit antiserum to mouse thymocytes, or no treatment. Cyclophosphamide (Cytoxan, Mead Johnson) was dissolved in sterile saline immediately before use and injected by the intraperitoneal route as follows: 125 mg/kg on day 3, 50 to 75 mg/kg on day 8, and 50 mg/kg on days 12 and 17. This schedule resulted in less than 10 percent mortality in controls inoculated intracerebrally with 1 percent homogenate of normal suckling mouse brain instead of virus. That cyclophosphamide was indeed immunosuppressive in these experiments was indicated by its effect on serum antibody titers. Neutralizing antibody to Theiler's virus was determined by standard plaque reduction technique with antibody considered present when there was a 50 percent reduction in plaque-forming units (PFU). There was a threefold reduction in the mean antibody