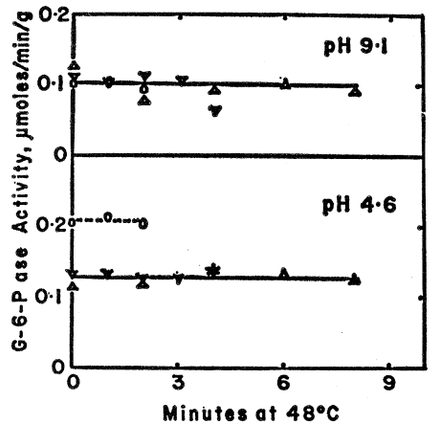

Fig. 3. Release of acid and alkaline phosphatases in the presence of $MgCl_2$ at various temperatures (○, 0°C; △, 24°C; ▽, 48°C). Those suspensions chilled to 0°C were subsequently treated at 48°C for 0, 1, and 2 minutes.

body technique (*6*). In the case of *E. coli* A, both acid and alkaline phosphatases are removed promptly and essentially independently of time and temperature, even in the presence of 0.01*M* $MgCl_2$ (Fig. 3) (*7*). That the amount of acid phosphatase obtained at 0°C is greater than that obtained at higher temperatures is reminiscent of the unexpected release of α-glucosidase from *Bacillus subtilis* at 0°C (*8*). The structural inferences from these results are consistent with cytochemical localization of alkaline phosphatase at the outer surface of the wall, and of acid phosphatase in a continuous compartment beneath the wall (*9*). Possibly, the location of acid phosphatase may be better described as being on a metabolically stabilized "plug" or "stopper" at an entry to the periplasm. Other factors must be involved because alkaline phosphatase is also described as being located in an intermediate layer of the wall or confined to the periplasm by the wall (*10*). Since warm-water treatment is not as effective with cells grown in enriched medium as with those grown in synthetic medium, some cellular fragility may have been encountered in my experiments. Less wall structure may have been deposited during growth in synthetic medium with the result that structural stability was decreased and the embedment of alkaline phosphatase within the wall was shallower.

With loss of compartmentalization, products of enzymic degradation are observed; among them, inorganic phosphate, acid-soluble nucleotides, and substances reacting with the Folin-Denis phenol reagent.

The attempts to localize cellular components according to osmotic criteria will undoubtedly yield useful information, especially by associating components with similar kinetics of release. Osmotically sensitive compartments outside of the membrane are inferred because certain components behave as though they are located between an osmotically driven surface and a porous limiting barrier. Although no direct evidence is offered, these components may have been located within the periplasm and mesosomes. Other components appear to be located outside of these structures, presumably on or within the wall. To some extent, the ease of release of surface components should correspond to the depth of localization within the surface structures external to the osmotic barrier. Structural inferences from this type of information will have to be correlated more closely with available cytological evidence (*11*).

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4. Cells of *E. coli* A in the logarithmic phase of growth were suspended at a concentration of 10 mg of dry weight per milliliter of 0.1*M* tris(hydroxymethyl)aminomethane-acetic acid, *p*H 7.3 buffer. After 1 hour at room temperature, the cells were harvested and resuspended in buffer warmed to 48°C. At intervals, samples were transferred to equal volumes of buffer chilled in ice. Extracts were analyzed for protein with a turbidity method (*3*). No nucleic acid precipitable in acid was observed.
5. Warm-water treatment was conducted with 0.01*M* buffer containing 0.01, 0.09, and 0.49*M* sucrose to provide for osmolarities of 0.021, 0.101, and 0.501, respectively.
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7. Phosphatase assays were performed at both *p*H 4.6 and *p*H 9.1 with 0.005*M* glucose-6-phosphate in 0.5*M* tris-acetate buffer for 2 hours at 37°C. Inorganic phosphate was determined by the method of R. L. Dryer, A. R. Tammes, J. I. Routh [*J. Biol. Chem.* **225**, 177 (1957)].
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Lipoperoxidation of Lung Lipids in Rats Exposed to Nitrogen Dioxide

Abstract. *Absorption spectra characteristic of diene conjugation and typical for peroxidized polyenoic fatty acids can be induced in rat lung lipids after the rats have been exposed to a scant amount of nitrogen dioxide (1 part per million) for 4 hours. The peroxidative changes do not occur immediately but appear to reach a maximum between 24 and 48 hours after exposure. The prooxidant effect of this atmospheric pollutant in rat lung lipids may be partially prevented by prior treatment of the animal with large doses of alpha-tocopherol.*

Nitrogen dioxide (NO_2) is one of several toxicants present in our atmosphere. It is among the most toxic of the oxides of nitrogen and is an important and integral component in the complex of chemicals producing photochemical smog. Evidence has indicated that the effects of nitrogen dioxide on man and lower animals are confined primarily to the respiratory tract. Histological studies revealed that lung mast cells undergo considerable change when animals inhale NO_2 (*1*). Therefore, we have studied the effect of NO_2 on the lipids of rat lungs.

Young Sprague-Dawley rats (weighing 175 to 200 g and maintained on stock diet of Purina Chow) were used. In certain experiments, six rats were exposed to NO_2 [1 part per million (ppm) by volume] for a single 4-hour period (*2*), whereas in other experiments they were exposed for 4 hours daily during a 6-day period. After the initial 30 minutes, the NO_2 concentration in the chamber was maintained between 0.9 and 1.1 ppm. Comparably treated control animals were forced to inhale ambient air in the chamber for a similar 4-hour period immediately after exposure to NO_2. For the multiple exposures, ten rats were placed in a stainless steel and glass chamber (0.4 m^3) provided with 30 air changes per hour and laminar flow. During the exposure periods, the control animals were forced to inhale ambient air in an equivalent chamber. The NO_2 was metered into the incoming ambient air from a pressurized cylinder containing 1500 ppm in nitrogen. The NO_2 concentration inside the exposure chamber was continuously monitored with an instrument calibrated with respect to

modified Saltzman reagent (*3*). Three to six samples were withdrawn during the exposure period for analysis by the reference method (*4*). During all exposure periods, the concentrations of NO_2 were in the range of 0.94 to 1.25 ppm. Concentrations of NO_2 in the control chamber ranged from < 0.01 to 0.08 ppm. Chamber temperatures varied between 26° and 28°C, and the relative humidity was between 39 and 48 percent. The carbon monoxide concentration was usually between 1 and 10 ppm, and the particulate concentration varied from 46 to 115 $\mu g/m^3$.

Lipid peroxidation was indicated by an intense ultraviolet absorption peak at 230 to 235 mμ, which, according to Bolland and Koch (*5*), represents the formation of a conjugated diene resulting from the peroxidation of linoleate, arachidonate, or other methyl-interrupted unsaturated fatty acids. There is also a secondary peak or shoulder with an absorption maximum at 260 to 280 mμ which possibly arises from the formation of ketone or aldehydic dienes. Lipoperoxidation in vivo in liver microsomal lipids was detected (*6*) in rats to which carbon tetrachloride was administered and in the fatty livers of weanling rats that were fed orotic acid (*7*).

Exposure of methyl linoleate to nitrogen dioxide (Fig. 1) produced more extensive peroxidation than exposure to air for the same time period produced. However, both produced the characteristic diene-conjugation absorbance at 233 mμ and the broader shoulder at about 278 mμ. Six hours after exposure to nitrogen dioxide (Fig. 2), there was little evidence of peroxidative changes. However, by 18 hours, peroxidative changes were apparent, and by 24 hours they seemed to have reached a maximum which was maintained for at least an additional 24 hours.

Because it seemed possible that the peroxidative changes might have occurred during processing of the lipid for spectral analysis, an antioxidant—butylated hydroxytoluene (0.1 percent)—was added to the extracts (at the time of the initial lipid extraction) of lung from some of those animals killed 36 and 48 hours after exposure. The presence of the antioxidant did not prevent the spectral changes previously observed, an indication that the peroxidation must have taken place in vivo and before extraction.

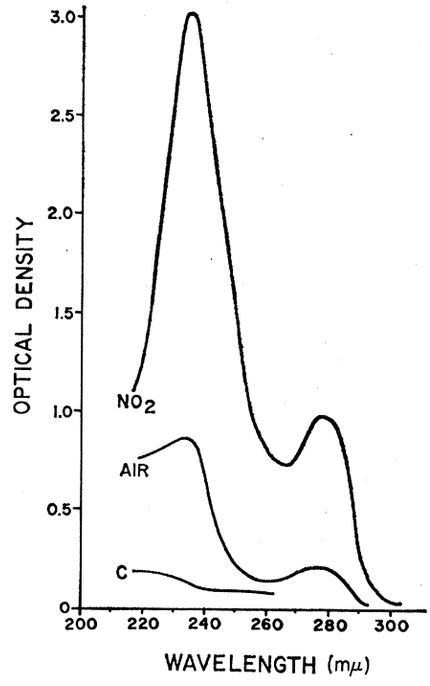

Fig. 1. Difference spectra for (i) methyl linoleate in absolute methanol ($0.15 \times 10^{-7}M$) exposed to NO_2 at a concentration of 1 ppm for 24 hours and (ii) an equivalent sample of same concentration exposed to ambient air for 24 hours. *C*, nonoxidized methyl linoleate.

To determine whether an antioxidant, such as vitamin E (*dl*-α-tocopherol) would prevent the peroxidative effects of nitrogen dioxide inhalation in vivo, we primed young rats (4 animals per group) for 3 days with *dl*-α-tocopherol in vegetable oil (10 mg per day). After this we divided them into two groups; one group continued to receive the α-tocopherol, and the other received only the vegetable oil. The animals were then placed for 4 hours on each of 6 consecutive days in an exposure chamber where the concentration of NO_2 was 1 ppm. We administered (by way of the mouth) vegetable oil without α-tocopherol to a control group of rats and placed them in an adjacent chamber for 4 hours per day; there they inhaled ambient air at a flow rate equivalent to that of the experimental group. Three animals from each group were killed immediately after the 6th day of exposure, and one from each group was killed 24 hours after the sixth or last exposure.

Lung lipid from rats that were exposed to nitrogen dioxide but which did not receive α-tocopherol showed more evidence of diene conjugation than lipid from the control group or from those receiving the antioxidant did. However, α-tocopherol, even in the abnormally large quantities administered, was only partially effective in preventing the lipid peroxidative changes induced by nitrogen dioxide. The broad peak of the difference spectra from these rats (Fig. 3) suggests that the longer exposure to nitrogen dioxide (4 hours per day for 6 days) produced more extensive and probably cumulative oxidative changes than the single 4-hour exposure did (Fig. 2). There were no differences in spectral changes between those animals killed immediately and those killed 24 hours later.

Light and electron microscopy reveal that the chemical changes in lung lipids induced by nitrogen dioxide are

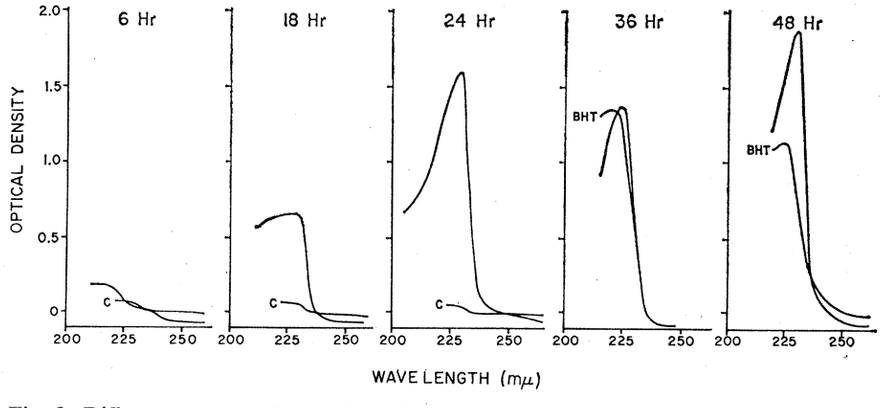

Fig. 2. Difference spectra for rat lung lipids (0.4 mg/ml) extracted and examined for ultraviolet absorption (cuvette with a 1-cm light path); after the rats had inhaled 1 ppm of nitrogen dioxide for 4 hours, they were killed at intervals. The difference in optical density at peak absorption between the lipids of exposed rats and those of controls is a measure of diene-conjugation absorption of the peroxidized lipid. *BHT*, butylated hydroxytoluene; *C*, absorption of lung lipids from rats exposed to ambient air.

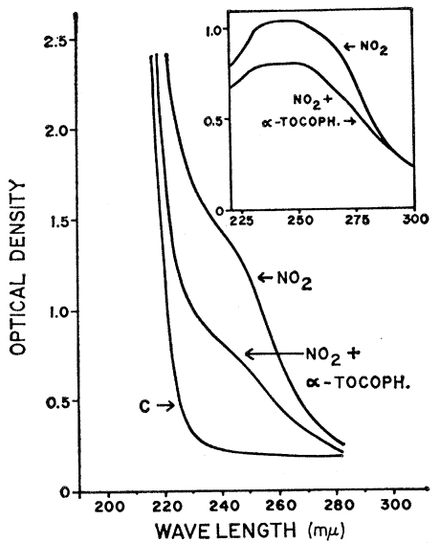

Fig. 3. Ultraviolet spectra for lung lipids (0.4 mg/ml) after inhalation of NO_2 (concentration 1 ppm) 4 hours daily for 6 consecutive days with respective controls. Inset: difference spectra obtained between peroxidized lipid and an equivalent quantity of nonperoxidized lipid. *NO_2*, exposed to nitrogen dioxide without α-tocopherol supplement; *NO_2 + α-tocoph.*, exposed to nitrogen dioxide with α-tocopherol supplement; *C*, exposed to ambient air and without α-tocopherol supplement.

also accompanied by characteristic structural change. We believe that this is the first time that chemical changes of this nature in animals exposed to such low concentrations of nitrogen dioxide have been reported.

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Circadian Oscillation Controlling Hatching: Its Ontogeny during Embryogenesis of a Moth

Abstract. *Populations of eggs of the moth* Pectinophora gossypiella *develop a circadian rhythm of hatching activity under certain circumstances. This rhythm derives from circadian oscillations in each egg, which can be initiated or made synchronous by steps or pulses of either light or temperature, but only if these signals are administered after the midpoint of embryogenesis. Correlations between the development of the oscillation, appearance of a pigment, sensitivity to photoperiodic induction, and a possible effect of light on growth rate are discussed.*

The moth *Pectinophora gossypiella* is subject to a photoperiodically inducible diapause in the fourth larval instar. It is easily reared on an artificial medium in the laboratory and has consequently become a useful object for the study of the photoperiodic time-measurement. In attempting to determine how circadian oscillations effect the measurement of night length in this species, we have recently sought and found three easily studied circadian rhythms in the egg, pupa, and adult. We here describe the rhythm found in the egg and report that the (cellular) oscillation responsible for the rhythm does not develop until midway through embryogenesis.

Stocks are maintained according to methods developed and described by Adkisson (*1*). They are kept at 26°C on a light-dark cycle of 14 hours of light (about 220 lumens of cool white fluorescent light per square meter) and 10 hours of dark (LD 14 : 10). Eggs are collected from parents kept in lucite boxes (13 by 19 by 9 cm) and fed sugar water (20 percent sucrose). A fine-mesh copper screen covers a square opening (8 cm^2) in the ceiling. Black velour paper (velour surface down) pressed against the screen provides an attractive surface for oviposition. Each night several thousand eggs are laid on the paper. They are oval (about 0.5 mm long and 0.25 mm wide), straw-colored, and only slightly opaque. At 20°C, embryogenesis lasts about 9 days, after which the small, first instar larva hatches.

The time of hatching in samples of the eggs collected in one night is assayed as follows. Egg-laden paper (300 to 400 eggs) is pasted to a carrier which in turn is placed on a fraction-collector device consisting of a lucite disk (33 cm in diameter) in which there are 24 slots, near the periphery, each surrounded by an adhesive cement (*2*). The egg carrier fits into these slots. Newly hatched larvae promptly explore the immediate neighborhood and are trapped in the adhesive. Every hour the egg carrier is automatically transferred to the next slot. Each day the lucite disk is replaced, and the trapped larvae are counted.

The night's egg-laying primarily occurs in the first few (about 4) hours (*3*). This small initial asynchrony in development within the sample is amplified throughout the next 10 days by variation in individual developmental rates and hatching behavior. Hatching of populations raised in a constant temperature and constant light (LL) is aperiodic, occurring over about 52 hours; the median developmental time in LL at 20°C is about 244 hours (Fig. 1A). A similar aperiodic distribution of hatching occurs in samples raised at 20°C in constant dark (DD), but both the median developmental time, about 275 hours, and the range of the distribution are much greater (Fig. 1A). The faster development in constant light may reflect a genuine effect of light on growth rate but we cannot yet confidently exclude the possibility that the weak illumination from the water-jacketed fluorescent lights slightly increases the temperature of the eggs. Light does have a direct action on the egg, promoting hatching when the animal is otherwise ready (see below). We believe that the reduced variance on developmental time in constant light is caused by that direct action.

In a light-dark cycle of LD 12 : 12, hatching is partitioned into discrete packets, one per day, with the median of each distribution coming soon after dawn (Fig. 1B). The eggs in the four cultures shown were laid in the same 10-hour night period; the phase of the four light regimes is staggered by 6-hour intervals. It is as though a portion of each light period constituted a "gate" (*4*) in which hatching could occur. The earlier the first gate relative to the ungated distribution in LL, the smaller is the number of eggs ready to