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Carbon Dioxide Production by Individual Mice as an Index of Behavioral and Metabolic Activity[1]

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Carbon Dioxide Production in Individual Mice as an Index of Behavioral and Metabolic Activity. BUSHNELL, P. J., EVANS, H. L., AND PALMES, E. D. (1985). *Fundam. Appl. Toxicol.* 5, 962–970. A noninvasive method for simultaneous real-time determination of spontaneous locomotor activity (LA) and CO_2 production (as minute volume expired CO_2, or $\dot{V}_ECO_2$) in mice is described. As an apical measure of overall metabolic activity, $\dot{V}_ECO_2$ proved sensitive to changes in many physiological processes, including behavioral activity. In normal mice, LA (as photobeam breaks) and $\dot{V}_ECO_2$ were positively correlated ($r = 0.73$), and stable over repeated daily tests. Food deprivation for 18 hr overnight increased LA while decreasing $\dot{V}_ECO_2$. Stimulation of peripheral sympathetic activity, either by exposure to cold air or by injection of epinephrine, increased $\dot{V}_ECO_2$ without affecting LA. Intoxication with pentobarbital produced a biphasic change in both measures: anesthetic doses increased LA and $\dot{V}_ECO_2$ during induction and recovery, and reduced them during the period of anesthesia itself. These results characterize $\dot{V}_ECO_2$ as an easily quantifiable, composite index of behavioral and metabolic activity in mice. This measure, along with its covariation with LA, may provide better information about toxic effects than any single screening test.

Screening procedures for pharmacological potency and/or chemical toxicity often measure behavioral activity in experimental animals. However, activity can be measured in many different ways, and the results obtained are often specific to the type of apparatus used (e.g., Weasner *et al.*, 1960; Gross, 1968; Tapp *et al.*, 1968; Finger, 1969). This specificity obtains because the type of measure employed defines a subset of the animal's total behavioral repertoire, and different subsets of this repertoire may respond differently to toxicants (e.g., Reiter, 1978).

A more comprehensive index of activity is provided by metabolic rate. This rate is an apical measure reflecting the sum of the basal metabolic rate (BMR) plus metabolism supporting overt behavior and the digestion of food (White *et al.*, 1968). Sleep disturbances, fatigue, and other changes in behavioral activity are common early signs of intoxication in humans. Thus the simple test of metabolic rate described here may provide an animal model of these effects of toxicants on human health.

The metabolic rate of an animal can be determined easily by measuring its CO_2 production (minute volume expired CO_2, or $\dot{V}_ECO_2$), using ir spectrometry to quantify the increase in CO_2 concentration produced by respiration over the ambient level of about 300 ppm. As an apical measure, $\dot{V}_ECO_2$ should respond to changes in a broad range of physiological parameters such as metabolism of a toxicant, food consumption, CNS activity, muscle tone, metabolism of brown adipose tissue, respiratory quotient, and ventilation rate and/or volume. Indeed, the power of $\dot{V}_ECO_2$ as a toxicant screen lies precisely in its potential ability to detect a response to intoxication at any of these

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levels in a single test. Further tests, beyond the scope of a screening procedure, would be required to pinpoint the site and mechanism of action of the compound.

Measurements of metabolic rate have been used to study feeding and body weight maintenance (Rothwell and Stock, 1980; Boyle *et al.*, 1981; Sugano, 1983), chronobiology (Stupfel *et al.*, 1980; 1981), and the metabolic effects of inhaled carbon monoxide (Stupfel *et al.*, 1979) and Formalin vapor (Jaeger and Gearhart, 1982). Thus, Stupfel *et al.* (1979) continuously recorded the $\dot{V}_E CO_2$ of groups of mice to demonstrate that the response to hypoxic challenge differed as a function of the time of day. In these nocturnal animals, $\dot{V}_E CO_2$ was maximal at midnight and minimal at noon. Exposure to 300 ppm CO at noon had no effect on the $\dot{V}_E CO_2$, while the same exposure at midnight reduced $\dot{V}_E CO_2$ by about 25%.

Jaeger and Gearhart (1982) observed that repeated 2-hr exposures of 15 ppm Formalin vapor suppressed ventilation rate, tidal volume, and $\dot{V}_E CO_2$ in mice. Indeed, metabolic rate was suppressed sufficiently to reduce rectal temperature by 3°C after 2 hr. The authors suggested that respiratory rate was suppressed to reduce exposure to this irritant gas, in a manner similar to that described by Alarie (1966, 1981) for a variety of airborne chemical irritants, and that this reduction in ventilation required a concomitant reduction in metabolic rate as well.

We now describe a system to measure $\dot{V}_E CO_2$ in individual mice in an unrestrained, unanesthetized state simultaneously with spontaneous locomotor activity (LA), an endpoint commonly used in behavioral pharmacology and toxicology. We compared some characteristics of $\dot{V}_E CO_2$ with LA, as measured by photobeam interruptions, including the response to several pharmacologic and environmental challenges. The potential of $\dot{V}_E CO_2$ as an index of the toxicity of inhaled toluene has been explored elsewhere (Bushnell *et al.*, 1984, 1985).

MATERIALS AND METHODS

Subjects

Adult male C57BL/6J mice,[2] 20–30 g in weight, were housed in groups of four in acrylic cages (13 × 28 × 17 cm) on pine chip bedding.[3] Except when removed for testing, all animals lived in a room automatically maintained on a 12 hr:12 hr light:dark cycle with light onset at 6 AM. The room was ventilated with a one-pass air supply, with 12–15 air changes per hour, the temperature maintained at 27 ± 1°C and a relative humidity between 45 and 65%. Rodent lab chow[4] and water were available *ad libitum*. Animal care practices conformed to standards promulgated by NIH (1980).

Apparatus

Mouse test chambers. Each of eight acrylic plastic cylinders (15 cm i.d. × 15 cm length, volume = 2.6 liters) was fitted with a galvanized mesh floor (15 × 15 cm) to support a mouse above absorbent paper and waste (Fig. 1). Air was drawn directly from the building ventilation system into the mouse chambers, thus avoiding possible contamination with CO_2 from laboratory personnel. This air entered each chamber at the top and was pumped out through two openings, at front and back, below the level of the grid floor. These airstreams were mixed, filtered, and pumped through the analyzers (see below). For long-term observations, water bottles and food pellets were added as shown in Fig. 1. Eight 2.8-W lamps (4 red for "dark" and 4 white for "light") maintained uniformity between lighting under testing and housing conditions. An exhaust fan maintained a constant airflow through the isolation cubicle (independently of the airflow through the mouse test chambers), creating masking noise within the cubicle and regulating the temperature at 27 ± 1°C.

Locomotor activity detection. Each chamber was bisected by an infrared photobeam, generated by an integrated LED/detector unit[5] (Fig. 1). The ir beams for two adjacent chambers were reflected off a polished aluminum strip mounted vertically between the two chambers.

Gas flow and analysis. The gas flow system had two parallel channels, each of which consisted of four mouse chambers, various flow control devices, an infrared CO_2 analyzer,[6] and an integrating chart recorder[7]; one of these channels is depicted schematically in Fig. 2. All fittings

[2] Jackson Labs, Bar Harbor, Maine.

[3] Beta Chip, Northwestern Products, Warrensburg, N.Y.

[4] Ralston Purina, St. Louis, Mo.

[5] Model 1100, Autotron, Danville, Ill.

[6] LIRA Model 303, Mine Safety Appliances, Pittsburgh, Pa.

[7] Model 252A, Linear Instruments, Reno, Nev.

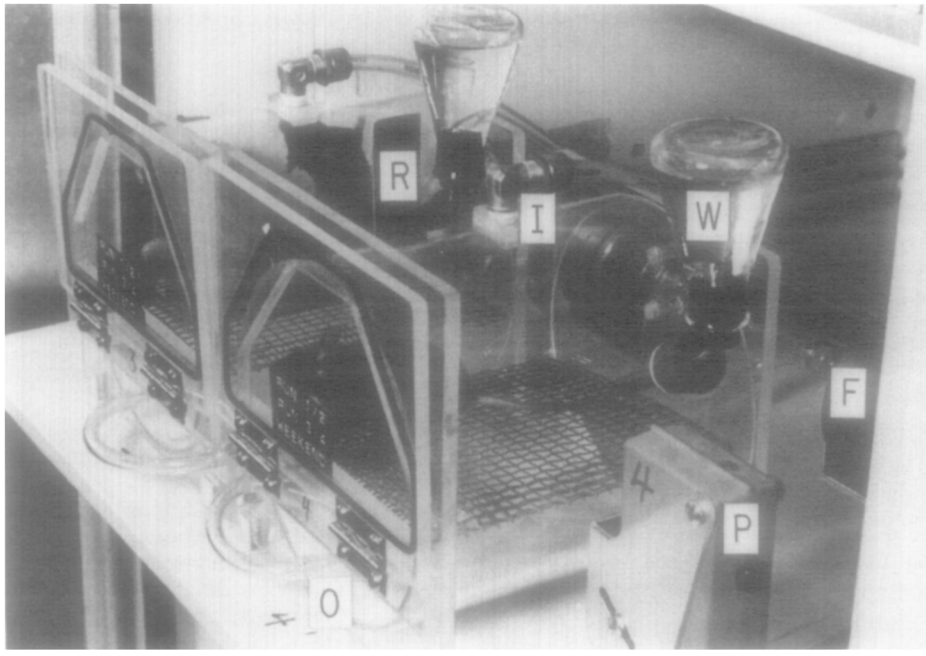

FIG. 1. Detail of two mouse test chambers mounted on a shelf in the isolation cubicle. I, air inlet; O, front air outlet (rear outlet not visible); F, particulate filter; W, water bottle (25 ml); P, ir photocell; R, reflector for ir photobeam.

and valves were brass; plastic Tygon tubing was 3/16-in. i.d. Air leaving each chamber passed through a solenoid valve, which directed it either to the CO_2 analyzer or directly to the exhaust pump[8] via a bypass line. One chamber was sampled at a time; gas from the other three chambers was discarded. Sampling parameters were determined in data collection software implemented by a minicomputer[9] via a SKED interface.[10] Each mouse chamber was sampled for 1.5 min, the first 15 sec of which were ignored. Thus, each mouse was sampled for 1.25 min in each 6-min cycle.

Gas flow through the chambers was controlled by critical orifices (23-gauge hypodermic needles) at 1.3 liters/min through both analyzer and bypass lines, producing one half air change per minute in the chambers. Span (0.20% CO_2 in N_2, certified standard) and zero (100% N_2, high purity) gases were used to calibrate the system. Because the CO_2 analyzer was sensitive to pressure, calibration gas pressures were adjusted with metering valves to be equal to that present under sampling conditions (4–6 cm H_2O vacuum).

The output of the CO_2 analyzer was integrated by the chart recorder, which produced switch closures at a frequency proportional to the CO_2 concentration in the airstream. The rate of CO_2 production was calculated from this digitized signal, using standard curves relating zero and span gas concentrations to integrator counts. Ambient CO_2 levels were measured daily (average = 310 ppm) and were very stable from day to day. The net CO_2 concentration from each mouse chamber was calculated by subtracting the ambient CO_2 from the total CO_2 leaving each mouse's chamber. Net CO_2 concentrations (ml/liter) were integrated over 1.25-min intervals and converted to $\dot{V}_ECO_2$ (ml/min) by multiplying by the total gas flow (1.3 liters/min). Finally, these $\dot{V}_ECO_2$ values were corrected for differences in metabolic mass by dividing by the 0.75 power of the animal's body weight, in kilograms (Kleiber, 1947).

Statistical analysis. Statistical analyses employed the analysis of variance and covariance programs of the BMDP package (Dixon, 1981). LA data were first transformed to their square roots to normalize the distributions of the scores. $\dot{V}_ECO_2$ data were not transformed. Post hoc comparisons between groups were done by Dunnett's procedure to compare treatment means with a control (Myers, 1966), or by matched-pair t tests in the case of repeated measures. The criterion for statistical significance was $p < 0.05$.

[8] Gast Model DOA-104-AA, SGA Scientific, Bloomfield, N.J.

[9] PDP/8a, Digital Equipment, Maynard, Mass.

[10] State Systems, Kalamazoo, Mich.

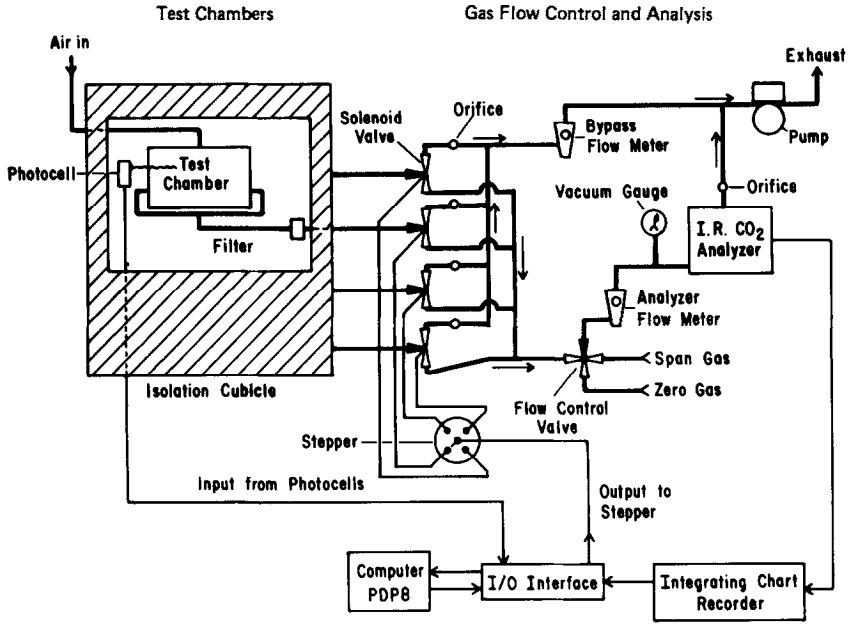

FIG. 2. Schematic diagram of the apparatus: one of two gas flow channels, each monitoring four test chambers, is shown. One chamber is depicted in the isolation cubicle. Air from one chamber at a time was pumped through the CO_2 analyzer; air from the other three chambers was discarded via the bypass line. The computer and the I/O interface controlled the state of each solenoid valve to determine which chamber to sample. Span and zero gases could be introduced directly into the CO_2 analyzer via the (manual) flow control valve for calibration. The vacuum gauge was used to monitor the gas pressure in the CO_2 analyzer.

PROCEDURES AND RESULTS

1. Diurnal Rhythms

Diurnal changes in LA and $\dot{V}_E CO_2$ were determined for eight mice adapted to the test chambers 24 hr prior to the start of data collection. Food and water were always available. Data collection began at 1300 hr and stopped at 1000 hr the following morning. Lights in the isolation cubicle changed from white (light) to red (dark) at 1800 hr and back to white at 0600 hr, in phase with the L:D cycle in the colony room. Calibration curves and body weights were determined immediately following data collection.

Diurnal changes in LA and in $\dot{V}_E CO_2$ were parallel over the 21-hr observation period (Fig. 3). Levels of both parameters climbed gradually during the 3 hr preceding darkness, reached a peak after 3 hr of darkness, and declined steadily thereafter.

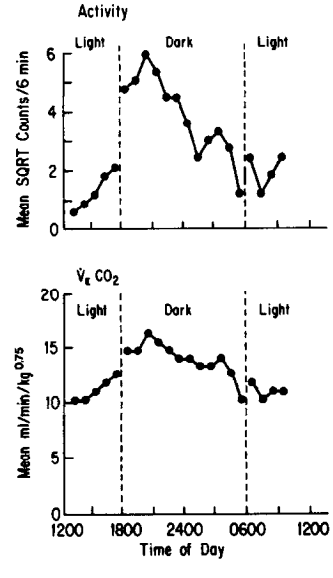

FIG. 3. Diurnal rhythm in LA (top) and $\dot{V}_E CO_2$ (bottom) obtained over a 21-hr observation. Data points indicate mean LA or $\dot{V}_E CO_2$ during the hourly interval bracketing the point shown.

The remaining tests were 72 min in length and were run during the light (inactive) phase of the light cycle. Mice were captured, weighed, injected (see below), and placed individually into test chambers without food or water. Data collection began as soon as the CO_2 concentrations in the first pair of chambers to be sampled had reached a plateau (about 1 min). The system was calibrated daily. All remaining tests were run using this procedure.

2. *Baseline Behavior*

To characterize the normal mouse's response to the apparatus and test procedures, 16 mice received short tests for 15 of 19 consecutive days (3 weeks, omitting intervening weekends). The correlation between LA and $\dot{V}_ECO_2$ was determined from the scores of the 16 mice averaged over a single session.

LA and $\dot{V}_ECO_2$ declined consistently as a function of time within each session (Fig. 4). LA decreased more within each session than did $\dot{V}_ECO_2$. Typically, LA of the least active animal fell to zero by 30 min, and at least half the animals showed zero activity by the end of the 72-min session. Coefficients of variation in LA scores always exceeded those of $\dot{V}_ECO_2$ scores; thus, toxic responses may be more easily detected by changes in $\dot{V}_ECO_2$ than by changes in LA. By comparison, changes across days were far less pronounced: neither $\dot{V}_ECO_2$ nor LA changed more than 10% from day to day. This stability in response suggested that little adaptation to the apparatus occurred over repeated daily tests and that procedural acclimation would therefore be unnecessary.

LA and $\dot{V}_ECO_2$ were positively correlated in these baseline tests (Fig. 5). An r value of 0.73 indicated that LA accounted for about 50% of the variance in $\dot{V}_ECO_2$. Extrapolation of the linear regression to zero LA suggested a "resting" $\dot{V}_ECO_2$ of about 10 ml CO_2/min/$kg^{0.75}$ body weight, which agrees reasonably well with calculated values for a "resting" 30-g mammal (0.6 ml/min, or 8.8 ml/min/$kg^{0.75}$; Kleiber, 1947).

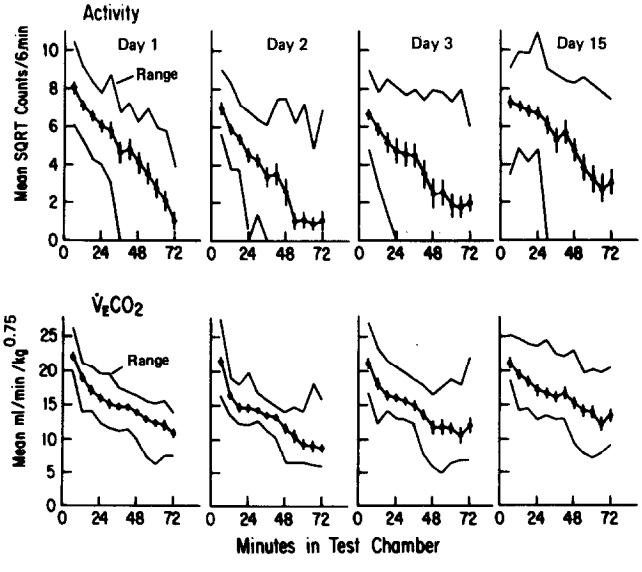

FIG. 4. Baseline behavior. Mean LA (top) and $\dot{V}_ECO_2$ (bottom) are shown for Days 1–3 and Day 15 of 15 daily 72-min tests. Vertical bars show ±1 SE. Lines above and below the means show the range of scores in each interval.

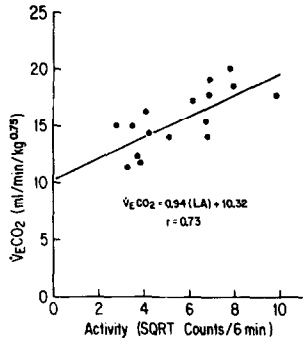

FIG. 5. Correlation between LA and $\dot{V}_ECO_2$. LA and $\dot{V}_ECO_2$ scores were averaged over a 72-min test for 16 untreated mice.

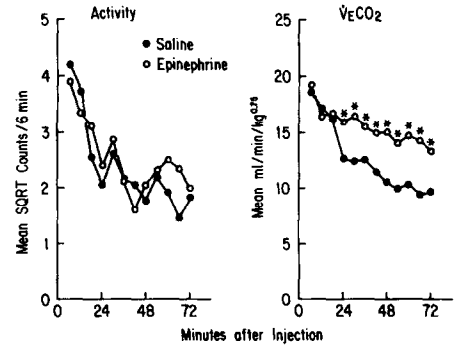

FIG. 6. Effect of epinephrine, 300 μg/kg, on LA (left) and $\dot{V}_ECO_2$ (right). Those points with asterisks differed significantly from corresponding control points.

3. *Response to Sympathetic Stimulation*

The sympathetic nervous system was stimulated (1) by injection of epinephrine and (2) by reduction of the ambient air temperature. Epinephrine in suspension[11] or physiological saline were injected sc into eight mice on alternate days, with half the mice receiving drug, and half saline, on each day. Doses were 15, 30, 50, 150, and 300 μg/kg, given to all mice in ascending order. The effect of ambient temperature was evaluated in two separate groups of eight mice each: one at 31°C (exhaust fan in isolation cubicle off) and the second at 18°C (established by cooling the interior of the isolation cubicle and the incoming air with crushed ice).

Epinephrine injections had no observable effect at doses up to 150 μg/kg. At 300 μg/kg, the drug still did not affect LA, but significantly [$F(1,7) = 6.51$, $p < 0.04$] increased $\dot{V}_ECO_2$ in all animals (Fig. 6). Analysis of a significant drug × interval interaction [$F(11,77) = 4.72$, $p < 0.008$] showed that $\dot{V}_ECO_2$ was increased at all but the first three time points.

Reduction of the ambient air temperature also increased $\dot{V}_ECO_2$ [$F(1,14) = 23.15$, $p < 0.0004$] without affecting LA (Fig. 7). Analysis of the significant interaction between temperature and interval [$F(11,154) = 6.02$, $p < 0.0001$] showed that $\dot{V}_ECO_2$ was significantly increased from 18 to 72 min after the beginning of the test.

4. *Effect of Food Deprivation*

Sixteen mice were deprived of food overnight (18 hr), with water always available. De-

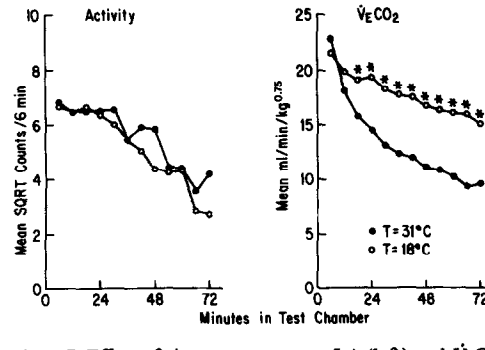

FIG. 7. Effect of air temperature on LA (left) and $\dot{V}_ECO_2$ (right). Asterisks as in Fig. 6 legend.

[11] Sus-Phrine (EPI), Berlex Laboratories, Wayne, N.J. For the 300 μg/kg dose, 450 μl fresh stock solution (5 mg EPI/ml in sterile water containing 10 mg sodium ascorbate, 6.6 mg sodium thioglycolate, 5 mg phenol, and 325 mg glycerin, with NaOH added to adjust pH) was added to 24.55 ml sterile saline to yield a concentration of 90 μg EPI/ml, which was injected in a volume of 0.10 ml/30 g mouse. In addition to 90 μg EPI, each mouse thus received 18 μg sodium ascorbate, 11.9 μg sodium thioglycolate, 9 μg phenol, and 585 μg glycerin.

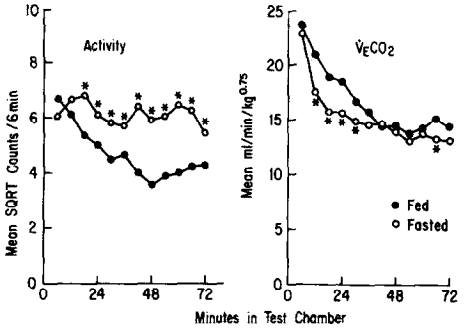

FIG. 8. Effect of 24-hr food deprivation on LA (left) and $\dot{V}_ECO_2$ (right). Asterisks as in Fig. 6 legend.

prived animals were significantly more active than fed animals [$F(1,15) = 29.37, p < 0.0001$] as they did not exhibit the within-session decline in LA normally observed in fed animals (Fig. 8). Analysis of the significant deprivation × interval interaction, [$F(11,165) = 3.51, p < 0.02$] showed that LA was increased in all intervals after 12 min. $\dot{V}_ECO_2$, by contrast, was significantly *decreased* in deprived animals [$F(1,15) = 5.47, p < 0.04$] but not at all time intervals. Analysis of the deprivation × interval interaction [$F(11,165) = 10.24, p < 0.01$] showed that $\dot{V}_ECO_2$ was reduced at time periods 12 through 30 min, and at 66 min after the start of the observation.

5. *Effect of Pentobarbital Intoxication*

To demonstrate intoxication with a CNS depressant, pentobarbital[12] or saline was injected ip into eight mice on alternate days, with half the mice receiving drug and the other half saline each day. All mice received the drug at 25 and 50 mg/kg, in that order.

Six minutes after injection with 25 mg/kg pentobarbital, both LA and $\dot{V}_ECO_2$ declined sharply for all animals and then rebounded to above control levels (Fig. 9). A dose of 50 mg/kg eliminated LA for 60 min, during which time $\dot{V}_ECO_2$ fell gradually below resting levels to a minimum at 30 min, then slowly recovered and overshot control levels as at the lower dose. LA began to reappear very shortly after $\dot{V}_ECO_2$ levels surpassed baseline levels.

DISCUSSION

These results demonstrate the utility of measuring $\dot{V}_ECO_2$ as an index of metabolic rate. In untreated mice, LA and $\dot{V}_ECO_2$ were highly correlated both in diurnal cycling and in response to repeated testing. Parallel changes in LA and $\dot{V}_ECO_2$ were also observed following pentobarbital injection. However, intervention by any of several other means uncoupled the metabolic response from LA, reducing the correlation between the two measures, and indicating that the combined measurement of LA and $\dot{V}_ECO_2$ provides more information than either one does alone.

The diurnal rhythms in LA and $\dot{V}_ECO_2$ in undisturbed mice (Fig. 3) are typical of noc-

[12] Pentobarbital (PNB) sodium, Abbott Laboratories, North Chicago, Ill. Fresh stock solution (50 mg PNB/ml in 50% sterile water, 40% propylene glycol (PG), 10% alcohol) was diluted with sterile saline to 7.5 (for 25 mg/kg) or 15.0 (for 50 mg/kg) mg/μl and injected ip in a volume of 0.10 ml/30 g mouse. In addition to sterile water, saline, and the PNB itself, each mouse thus received 7 μl PG and 2 μl alcohol at 25 mg/kg PNB and 14 μl PG and 4 μl alcohol at 50 mg/kg. The working solutions were kept refrigerated for the 2-day experiment.

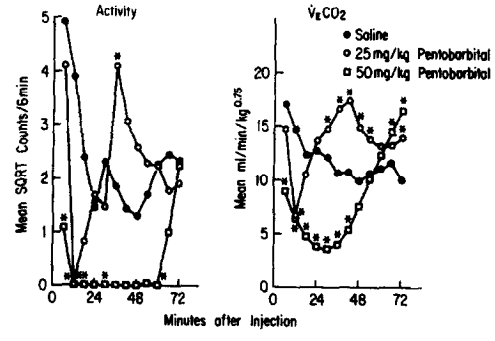

FIG. 9. Effect of pentobarbital on LA (left) and $\dot{V}_ECO_2$ (right). Asterisks as in Fig. 6 legend.

turnal animals. The detection of this rhythm in LA indicates the adequacy of this simple, one-photobeam system for quantifying locomotor behavior. Similar diurnal changes in $\dot{V}_ECO_2$ in mice and rats have been reported (Stupfel *et al.*, 1980, 1981). The parallel rhythms in LA and $\dot{V}_ECO_2$ suggest that the two indices of diurnal rhythmicity are directly coupled; nevertheless, the correlation between $\dot{V}_ECO_2$ and LA was less than one (Fig. 5), because (1) CO_2 production continues during "resting" when no measurable behavior occurs, (2) $\dot{V}_ECO_2$ also reflects changes in nonbehavioral physiological variables (e.g., digestion of food and heat production), and (3) some behaviors are not detected as photobeam interruptions. Thus, adding photocells to the system would probably not increase the correlation between $\dot{V}_ECO_2$ and LA sufficiently to justify additional cost and data reduction.

The high consistency of LA and $\dot{V}_ECO_2$ responses from session to session (Fig. 4) indicates that this test is influenced little by experience or conditioning. Both LA and $\dot{V}_ECO_2$ decreased with time after handling, regardless of preceding restraint or ip injections (compare Fig. 4 with control values in Figs. 6–9). This consistency of baseline is an important advantage for toxicology as short-term tests may be performed repeatedly without significant changes in response.

Several manipulations of the mouse's physiology caused divergent changes in LA and $\dot{V}_ECO_2$. Stimulation of the sympathetic nervous system by injection of epinephrine (Fig. 6) or by exposure to cold (Fig. 7) caused an increase in $\dot{V}_ECO_2$ without affecting LA. These results are consistent with known mechanisms for maintenance of body temperature under varying ambient temperatures (e.g., Hart, 1971) and the involvement of the sympathetic nervous system in this process (e.g., Himms-Hagen, 1967, 1972; Rothwell and Stock, 1980). During this 72-min session, mice apparently generated the additional heat necessary to maintain body temperature by means other than LA, e.g., by the metabolism of brown adipose tissue (Himms-Hagen, 1981). Such nonshivering thermogenesis would not be detectable by behavioral means, as this increased metabolism requires no overt behavioral activity.

Food deprivation significantly increased LA (Fig. 8), a response well documented in rodents (Richter, 1922; Evans *et al.*[13]) and attributable to generalized activation in place of foraging behaviors. Food deprivation reduced $\dot{V}_ECO_2$, thus uncoupling LA and metabolic rate. Food deprivation has been shown to decrease sympathetic activity as assessed by cardiac norepinephrine turnover (Young and Landsberg, 1977). Suppression of metabolic rate in response to starvation may have survival value by conserving energy reserves under conditions of hypoglycemia.

Intoxication with pentobarbital affected both LA and $\dot{V}_ECO_2$ in parallel (Fig. 9). In the deeply anesthetized state, LA was reduced to zero, and $\dot{V}_ECO_2$ descended to levels below the resting baseline (cf. Fig. 3). The $\dot{V}_ECO_2$ curve was a better indicator of the time course of the anesthesia than was overt behavior. The induction and recovery phases of anesthesia produced high levels of both LA and $\dot{V}_ECO_2$ because of temporary hyperactivity from incoordination and impaired motor control (Bushnell *et al.*, 1975).

Skeletomuscular behavior is a major determinant of metabolic rate in exercising animals (Pasquis *et al.*, 1970), but $\dot{V}_ECO_2$ reflects more than this behavior. The present data show that the mouse may change its behavioral and metabolic activity levels independently, e.g., to conserve heat, or in response to food deprivation or sympathetic stimulation. Changes in metabolic rate may provide important supplementary information on the response to environmental toxicants such as toluene (Bushnell *et al.*, 1985).

[13] H. L. EVANS, P. J. BUSHNELL, J. D. TAYLOR, A. MONICO, J. J. TEAL, AND M. J. PONTECORVO (1985). *A System for Assessing Toxicity of a Chemical by Continuously Monitoring of Homecage Behaviors.* Submitted for publication.

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