Effect of Acute and Chronic Stress on Amobarbital Metabolism in the Rat

JAMES EDWARD HUFF,* STANLEY M. SHAW, and JOHN E. CHRISTIAN

Abstract □ Labeled amobarbital was employed in an investigation of the utilization of urinary excretion of a labeled compound as an index for indicating altered amobarbital metabolism due to a stress. A study of the effect of an external acute unilateral hindleg ligation stress showed significant differences in amobarbital and/or metabolite urinary excretion patterns between stress and control groups. Adrenal ascorbic acid as an index of acute stress showed a significant difference between groups. Repeated injection of amobarbital as an internal chronic stress resulted in a significant difference between stress and control rat urinary excretion of amobarbital and/or metabolites.

Keyphrases □ Amobarbital-^{14}C metabolism—acute, chronic stress effect □ Adrenal ascorbic acid—acute stress index □ Stress, hindleg ligation—amobarbital-^{14}C metabolism □ Excretion, fecal, urinary—amobarbital-^{14}C □ UV spectrophotometry—analysis □ Scintillometry—analysis

The effect of various stress conditions or stressors on living organisms has been an area of increasing concern and activity in the scientific community. A recent review (1) listing 326 references defined stress "as the response of an organism to a variety of challenging and threatening events which inevitably occur and recur throughout life." These conditions of stress may be initiated by a variety of physical and/or mental stimuli which may include infections, wounds, burns, accidental injuries, exposure to toxic chemicals, noise, vibrations, extremes of temperature, prolonged or extreme physical activity, ionizing radiations, sleeplessness, pain, fear, anxiety, and other emotional strains (2). It is generally accepted that most stressors, if sufficiently intense and prolonged, initiate the general adaptation syndrome (3), with the successive stages of alarm reaction, adaptation, and exhaustion.

Information relating to the interaction between stress and drug effect is only in the embryonic stage. There exists a void in scientific knowledge, specifically pertaining to "what does happen" when a stressed individual receives a drug, the dose of which was titrated on normal subjects at the time of the drug's inception. Barry and Buckley (1) stated that the effect of stress may potentiate, counteract, or have no effect on the action of a drug, but that the variation in drug action, if any, should be known. Levy (4) recently stated that most pharmacokinetic studies are conducted on healthy ambulatory subjects. Levy suggested that many of these investigations should be carried out also on debilitated patients in order to assess the effect of various pathological conditions on the kinetics of drug absorption, distribution, and elimination.

Since barbiturates are centrally acting nervous system depressants used in conditions of mental and physical stress, an investigation of the effects of experimental stress on the pharmacology of barbiturates would seem to be of paramount concern. Therefore, the overall objective of this investigation was to follow the urinary and fecal excretion of amobarbital and metabolites as observed in stressed and nonstressed rats, using tracer techniques, in order to determine if an alteration in the process of drug metabolism occurs from stressor effects; and to observe if the alteration can be detected using urinary excretion as a quantitative index of stress. The selected methods of stress were short term unilateral hindleg ligation, designated as an external acute stress, and repeated intraperitoneal administration of the barbiturate, designated as an internal chronic stress.

EXPERIMENTAL

Animals—Male rats of the Sprague-Dawley strain[1] were used throughout this investigation. They were maintained on commercial laboratory chow[2] and water *ad libitum*. The animal quarters were air conditioned and kept at 21.1–22.1° (70–72°F.) at all times. As closely as possible, lighting was regulated to allow 12 hr. of uniform illumination and 12 hr. of darkness each day.

The animals were initially placed in large community cages in groups of five for a period of 7–14 days. Gentling and petting were performed twice daily to individual rats. After this adjustment period the animals were randomly transferred to individual metabolism cages. For 7–10 days, the animals were individually weighed and handled each morning. Moreover, a conditioning regimen (5) was followed for the rats used in the acute stress studies. In the conditioning program the animals received 0.5 ml. of physiologic saline intraperitoneally for 5 days prior to measurement of experimental parameters.

Liquid Scintillation Counting—A liquid scintillation spectrometer[3] was used for radioactivity determinations. An XDC scintillator (6) was used for counting urine samples since the scintillator solution can contain almost 30% water and still allow good counting efficiency for ^{14}C. The TC scintillator counting solution of Mahin and Lofberg (7) was modified[4] and used for ^{14}C fecal counting. Absolute disintegration rates were determined by internal standardization (8).

Amobarbital-^{14}C Assay—The radiochemical purity of amobarbital-2-^{14}C[5] was determined by TLC and autoradiography. A series of spots ranging from 1–100 mcg. of labeled amobarbital were spotted on commercial adsorbent (Adsorbosil-P-1) plates prepared in the usual manner and each developed in one of four solvent systems.[6] Autoradiograms of the developed plates were obtained with Kodak No-Screen X-ray film. In all cases only one spot was visible and R_f values for the series of spots from each of four solvent systems were identical. No radiochemical impurities were present. No differences in R_f value of radiochemical purity were observed between the acid form and a sodium salt of the amobarbital-2-^{14}C when spotted using alcohol, water, or urine as the dissolution vehicle.

[1] Sprague-Dawley, Inc., Madison, Wis.
[2] Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois.
[3] Packard Model 3003 Tri Carb with Packard Model 574 Automatic Control, Packard Instrument Co., Inc., Downers Grove, Illinois.
[4] The TC scintillator consisted of 5 parts toluene, 4 parts 2-ethoxyethanol, and 0.03% 2,5-diphenyloxazole (6 g./l. toluene).
[5] Supplied by Smith Kline & French Laboratories, Philadelphia, Pa., as 5-ethyl-5-isopentylbarbituric acid-2-^{14}C.
[6] Chloroform–acetone (9:1), benzene–acetic acid (9:1), dioxane–benzene–aqueous ammonia (20:75:5), acetone–*n*-butyl alcohol–ammonium hydroxide (9:9:2).

Chemical purity of the labeled compound was determined spectrophotometrically.[7] A series of five scan curves were attained from 350 to 190 mμ using standard and labeled amobarbital in concentrations of 25 to 1.6 mcg./ml. in 0.5 *N* sodium hydroxide solution. The Lambert-Beer law was obeyed and the resulting straight lines were similar. The scan curves were in agreement and exhibited identical maximum and minimum inflections at 253 and 232 mμ, respectively. Melting point values were attained using a Buchi melting point apparatus for standard amobarbital (157–159°) and amobarbital-2-^{14}C (158–159°). The amobarbital melting points were in good agreement with the official compendia (9).

Statistics—The Student's *t* test of significance between two sample means was used throughout the investigation. An analysis of variance using a two-factor experimental design with repeated measures on one factor was used with an *F* test to determine overall differences in each study. The level of significance used for all determinations between stress groups and control groups was $p < 0.05$.

Determination of Ascorbic Acid—Eight animals were subjected to unilateral hindleg ligation for 1.5 hr. while eight animals served as controls. Animals were sacrificed by cervical dislocation. The adrenals were rapidly removed, trimmed of fat, and frozen until assay. Less than 5 min. elapsed between removal of the rat from the metabolism cage until the adrenals were frozen. The method of Maickel (10) was employed for the determination of adrenal ascorbic acid. The study was conducted during the winter season.

External Acute Stress Program—An excretion study was undertaken during the winter season using unilateral hindleg ligation as the stress. Rupe (11, 12) showed that rats subjected to such stress exhibited a shortened duration of pharmacologic response to various drugs. Driever (13, 14) further demonstrated that stressed rats clear various drugs from the blood at an increased rate compared to controls. The stress was accomplished by wrapping a small unbroken rubber band (2 cm. in diameter) three times around the upper portion of the hindleg of the rat. A rapid swelling and purple discoloration of the leg took place. Hindleg ligation was applied to the stress group of 5 rats 1.5 hr. prior to intraperitoneal injection of amobarbital-2-^{14}C and removed upon injection. A group of five animals served as controls. A dose of 10.18 μc. (17.0 to 17.9 mg./kg.) of amobarbital-2-^{14}C was administered to each 380–400-g. stress and control rat in the form of a freshly prepared sodium salt. Urine was collected at numerous intervals for a period of 144 hr. Fecal samples were taken at intervals of 0–24, 24–48, 48–72, and 72–96 hr.

Internal Chronic Stress Program—During the winter season, 30 rats weighing between 205–234 g. were randomly divided into two groups; the stress group received an anesthetic dose of 35 to 40 mg./kg. of unlabeled sodium amobarbital intraperitoneally twice daily while the control group received normal saline twice a day. Following the 14 days of pretreatment with unlabeled amobarbital or normal saline, all stressed and control rats received 4.98 μc. (11.3 to 13.6 mg./kg.) of amobarbital-2-^{14}C. The intervals of urine collection were 0–2, 2–4, 4–6, 6–8, 8–10, and 10–12 hr. while feces were collected only for the 0–24 hr. interval. The rats were sacrificed 24 hr. after the injection of labeled drug by cervical dislocation. The entire liver from each rat was rapidly removed, rinsed in normal saline, blotted on a paper towel, and accurately weighed.

RESULTS AND DISCUSSION

Adrenal Ascorbic Acid Depletion—Mean values of 445 ± 46 and 605 ± 22 mg.[8] adrenal ascorbic acid per 100 g. (mg. %) of adrenal tissue weight for the stress and control groups, respectively, were obtained from this phase of the investigation. A greater depletion of adrenal ascorbic acid was observed in the ligated group than in the control group ($p < 0.01$), which indicated that a stress condition existed. The adrenal tissue weight of the stress and control groups was 35.38 ± 3.56 and 38.04 ± 3.54 mg., respectively. Although not statistically significant the adrenal tissue weight of the stress group was consistently lower than the control group.

External Acute Stress Urinary Excretion—Comparison between stress and control groups of the level of amobarbital and/or metabolites excreted at each urine collection interval showed that no statistical difference could be assigned to any specific time period. The cumulative mean percent of the administered dose of labeled amobarbital recovered from the urine as amobarbital and/or metabolites during the 0–144 hr. collection interval was 82.7% for the control group and 90.2% for the stressed group. The urinary excretion rate reached a plateau at the 0–8 hr. interval (Fig. 1) with 74.8% excreted for the nonstressed group and 81.8% for the stressed group. Since the two curves were relatively straight lines after the 10–12 hr. collection period, only the 0–12 hr. cumulative interval is shown in Fig. 1. The control and stress urinary excretion data for the 0–12 hr. interval were 78.8 and 86.5%, respectively. Statistically significant differences ($p < 0.05$) were observed between the two curves for the 0–12 hr. cumulative excretion period. During the 0–12 hr. collection interval the urine volume of the stress animals (7.9 ml./animal) did not differ significantly from the controls (7.2 ml./animal).

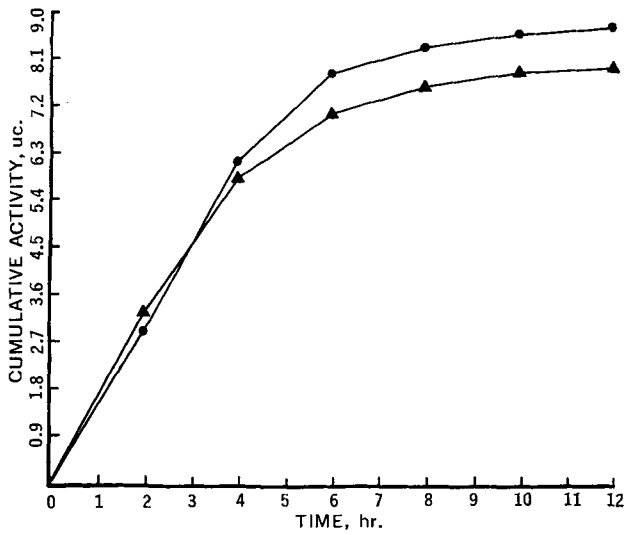

Figure 1—*The effect of an external acute stress on the cumulative urinary excretion of amobarbital and/or metabolites. Key: ●, stress group; ▲, control group.*

Internal Chronic Stress Excretion—Since most drugs are prescribed in multiple doses at regular intervals, an internal chronic dosage program was designed to test the more subtle type stress effect of repeated drug administration. Repeated administration of amobarbital was selected as the chronic stress even though the use of repeated amobarbital injections may confound the development of enzyme induction and tolerance to the particular drug in measuring effects on metabolism of the same drug.

The mean urine, fecal, and total excretion of amobarbital and/or metabolites for the stress group were 88.3, 10.2, and 98.5% of the

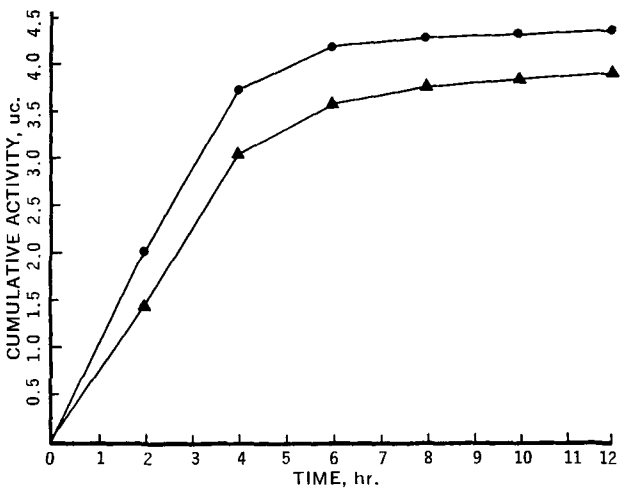

Figure 2—*The effect of an internal chronic stress on the cumulative urinary excretion of amobarbital and/or metabolities. Key: ● stress group; ▲, control group.*

[7] Beckman DK-2A Ratio Recording Spectrophotometer, Beckman Instruments, Inc., Fullerton, California.

[8] Results expressed as the mean ± standard error.

administered dose, while the values for the control group were 79.1, 11.1, and 90.2%. The mean cumulative urinary excretion plots for the stress and control groups (Fig. 2) indicate a higher cumulative urinary excretion level of amobarbital and/or metabolites for the stress group. An analysis of the stress and control data show a definite statistical difference ($p < 0.01$) between groups for the cumulative urinary excretion of amobarbital and/or metabolites. A statistical comparison of the individual time period urinary excretion data showed a significant difference ($p < 0.05$) between groups for the 6–8, 8–10, and 10–12 hr. collection intervals. During the 0–12 hr. collection interval the urine volume of the stress animals (7.0 ml./animal) did not differ significantly from the controls (6.5 ml./animal).

During the 2-week pretreatment period it was noticed that the stress rats exhibited a shorter and shorter sleep time response to the same dosage of amobarbital sodium, while the control rats were not noticeably affected by the saline injections. The sleep time observations were recorded only visually. Therefore, the following observations were only qualitative; but the trend of decreased sleep time was definitely noticeable. The injections of amobarbital caused immediate induction of sleep ($\leq$ 5 min.) during the first three days of treatment. Sleep induction time was initially (3 to 6 days) decreased, also noted by Aston and Hibbeln (15), in that the rats exhibited narcosis almost as soon as the needle was withdrawn. Subsequently, however, the induction time increased until the 9–11 day period in which more than one-half the rats only exhibited ataxia and not anesthesia. The final injection of the central nervous system depressant resulted in the majority of the rats remaining awake, while the ones which lost the righting reflex did so for only a brief period of time ($\leq$ 10 min.). Decreased sleeping time has been demonstrated by others (12, 16) after barbiturate administration to rats subjected to unilateral hindleg ligation. It is interesting to note that mean liver weights were recorded as 4.571 $\pm$ 0.087 and 4.478 $\pm$ 0.085 g./100 g. body weight for the internal chronic stress and control rats, respectively. The results indicated no differences between groups.

SUMMARY AND CONCLUSIONS

Tracer techniques were utilized in an investigation of the urinary excretion of amobarbital in stressed and nonstressed rats. Adrenal ascorbic acid depletion was used as an index of external acute stress induction. The adrenal ascorbic acid level of the hindleg ligation stress group was 445 $\pm$ 46 mg.% while that of the control group was 605 $\pm$ 22 mg.%. The significant difference of adrenal ascorbic acid levels between groups showed that a stress condition existed and that hindleg ligation as a stress was therefore applicable to the study of the utilization of urinary excretion as an index for indicating a stress condition. A study of the effect of an external acute unilateral hindleg ligation stress on the urinary excretion of amobarbital and/or metabolites showed significant differences in urinary excretion patterns between stress and control groups. An internal chronic stress study consisting of twice daily repeated injections of amobarbital for 14 days showed a significant difference in urinary excretion of amobarbital and/or metabolites between treated and nontreated rats. Changes in urinary excretion phenomena as a quantitative index of a stress-altered metabolic rate response in rats were shown to be applicable for the detection of the effects of external acute ligation stress and internal chronic repeated injection stress.

REFERENCES

(1) H. Barry, III and J. P. Buckley, *J. Pharm. Sci.*, **55**, 1159 (1966).

(2) W. H. Griffith, *Federation Am. Soc. Exptl. Biol. Conf.*, 1967, p. 3.

(3) H. Selye, *J. Clin. Endocrinol.*, **6**, 117(1946).

(4) G. Levy, *J. Pharm. Sci.*, **56**, 928(1967).

(5) C. Sanchez, T. S. Miya, and W. F. Bousquet, *Proc. Soc. Exptl. Biol. Med.*, **123**, 615(1966).

(6) G. A. Bruno and J. E. Christian, *Anal. Chem.*, **33**, 1216 (1961).

(7) D. T. Mahin and R. T. Lofberg, *Anal. Biochem.*, **16**, 500 (1966).

(8) C. H. Wang and D. L. Willis, "Radiotracer Methodology in Biological Sciences," Prentice-Hall, Englewood Cliffs, N. J., 1965, p. 131.

(9) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 40.

(10) R. P. Maickel, *Anal. Biochem.*, **1**, 498(1960).

(11) B. D. Rupe, Ph.D. thesis, Purdue University, 1963.

(12) B. D. Rupe, W. F. Bousquet, and T. S. Miya, *Science*, **141**, 1186(1963).

(13) C. W. Driever, Ph.D. thesis, Purdue University, 1965.

(14) C. W. Driever, W. F. Bousquet, and T. S. Miya, *Intern. J. Neuropharmacol.*, **5**, 199(1966).

(15) R. Aston and P. Hibbeln, *Science*, **157**, 1463(1967).

(16) W. F. Bousquet, B. D. Rupe, and T. S. Miya, *J. Pharmacol. Exptl. Therap.*, **147**, 376(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 12, 1969 from the *Department of Bionucleonics, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Accepted for publication September 29, 1969.

This investigation was supported in part by the National Center for Radiological Health, U. S. Public Health Service and conducted under the auspices of the Institute for Environmental Health, Purdue University.

* Present address: The University of Rochester, School of Medicine and Dentistry, Rochester, NY 14620