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Temporal Modulation of Nephrotoxicity and of Feeding and Drinking by Gentamicin Treatment in Rats

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ABSTRACT

Gentamicin-induced nephrotoxicity varies temporally, with a peak being observed when this antibiotic is administered during the resting period and a trough when given during the activity period of rats. These variations are modified by fasting and by restricted feeding schedules. In this study, food and water intakes of adult female Sprague-Dawley rats were measured during pre-treatment (days 1 to 5) and during treatment (days 6 to 10) with gentamicin (80 mg/kg/day, i.p.) injected at 1300 h or 0100 h. A significantly higher level of serum creatinine was observed when gentamicin was administered at 1300 h compared to 0100 h, and a significantly lower creatinine clearance was found in rats treated with gentamicin at 1300 h compared to those treated with saline at the same time. Gentamicin treatment at 1300 h or 0100 h resulted in a decrease in the 24 h food intake. In addition, in the gentamicin-treated group at 0100 h, the maximal food intake observed at late dark during the pre-treatment period decreased during treatment, and early dark rather than late dark maximal intake occurred. Our data demonstrate that gentamicin induces a nephrotoxicity that varies temporally, and that gentamicin treatment inhibits food intake and alters its nocturnal variations.

RÉSUMÉ

La toxicité rénale causée par la gentamicine varie dans le temps, avec un maximum lorsque cet antibiotique est administré durant la période de repos et un minimum durant la période d'activité du rat. Ces variations peuvent être modifiées par le jeûne ou en limitant les périodes d'accès à la nourriture. La présente étude a mesuré les ingestions de nourriture et d'eau de rates adultes Sprague-Dawley durant une période de pré-traitement (jours 1 à 5) et de traitement (jours 6 à 10) à la gentamicine (80 mg/kg/jr, i.p.) injectée à 1300 h ou à 0100 h. Le niveau de créatinine sérique était significativement plus élevé chez les animaux traités à 1300 h qu'à 0100 h, et la clairance de la créatinine était significativement plus faible chez les rates traitées à la gentamicine à 1300 h que chez celles traitées au salin à la même heure. Le traitement à la gentamicine à 1300 h ou à 0100 h a réduit la prise de nourriture de 24 h. Chez le groupe ayant reçu la gentamicine à 0100 h, l'ingestion de nourriture maximale observée à la fin de la phase nocturne durant la période de pré-traitement, a diminué durant la période de traitement pour devenir maximale au début de la phase nocturne. Ces résultats démontrent que la toxicité rénale de la gentamicine varie dans le temps, et que le traitement à la gentamicine réduit l'ingestion de nourriture et en altère les variations nocturnes.

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"Each friend represents a world in us, a world possibly not born until they arrive, and it is only by this meeting that a new world is born."

Anaïs Nin

CONTRIBUTIONS OF AUTHORS

The manuscript was written by the first author, Nancy Julien, under the guidance of the last author, Louise Thibault (thesis supervisor), fourth author, Denis Beauchamp (thesis co-supervisor), and third author Gaston Labrecque (research collaborator). It was revised by Dr. Thibault and Dr. Beauchamp, who gave constructive and helpful suggestions in improving both the format and contents of the manuscript which have led the manuscript to the final form. The second author, Meryem Karzazi, performed the cellular regeneration and gentamicin cortical accumulation assays and helped for the urinary enzymes activity assays. The third, fourth, and last authors also provided all the materials, supplies and facilities that were necessary to carry out the experiment. This research was supported by a grant from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR-soutien aux équipes de recherche) held by the third, fourth, and last author. Nancy Julien was the recipient of a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada.

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LIST OF ABBREVIATIONS

Abbreviations used throughout text:

AG: aminoglycosides

ALF: ad libitum feeding schedule

ANOVA: analysis of variance

AUC: area under the time-concentration curve

b.w.: body weight

dL: deciliter

DLAM: Diet Scan Lab Animal Monitoring System Software

DNA: deoxyribonucleic acid

dpm: disintegrations per minute

e.g.: for example

Fig: figure

g: gram

h: hour

i.e.: that is

i.m.: intramuscular

Inc.: incorporated

i.p.: intraperitoneally

i.v.: intravenous

kg: kilogram

L: liter

mg: milligram

min: minute

mL: milliliter

mM: millimolar

NAG: N-acetyl-ß-D-glucosaminidase

nm: nanometer

PAA: poly-L-aspartic acid

s.c.: subcutaneously

SEM: standard error of the mean

TRF: time-restricted feeding schedule

vs.: versus

βGal: β-galactosidase

γ-GT: γ-glutamyltransferase

μCi: microcurie

μg: microgram

μl: microliter

Chapter 1

INTRODUCTION

Aminoglycosides (AG) are large spectrum antibiotics that have been used in hospital settings for many years, alone or in combination with other antibiotics, to combat severe gram-negative bacterial infections. Their efficacy, however, is shadowed by the fact that all AG have the potential to induce renal toxicity as a side effect (Sande and Mandell, 1990). This nephrotoxicity occurs in 8% to 26% of hospitalized patients receiving these antibiotics (Kahlmeter and Dahlager, 1984), and although it is generally reversible upon drug discontinuation it complicates the patients' conditions, prolongs the hospital stay, and increases the medical costs (Sande and Mandell, 1990).

Numerous animal studies performed on rodents have shown the renal toxicity caused by AG to vary temporally; the peak of nephrotoxicity being observed in the resting period of the animals (during the day) and the trough corresponding to the activity period (at night) (Yoshiyama et al., 1992; Lin et al., 1994; Beauchamp et al., 1996; Lin et al., 1996; Yoshiyama et al., 1996a). The same phenomenon was observed in humans, as reported by Prins et al. (1997) who showed that the renal toxicity of AG was maximal in patients treated between 0100 h and 0730 h (resting period) compared to patients treated between 0800 h and 1530 h or 1600 h and 2300 h.

Since food and water intakes have a rhythmic nature, in which consumption occurs mainly during the rodent's and human's active period, the role the presence of food may play in modulating the temporal variations in nephrotoxicity caused by AG was investigated. A short fasting period (Beauchamp et al., 1996) or a limited feeding schedule (Beauchamp et al., 1997) modified the variations in renal toxicity normally observed in ad libitum fed animals. In these studies, the minimal toxicity was consistently observed when the animals were injected during the period where food was made

available. None of these studies, however, reported how much food and water were ingested during the access periods.

Thus, there were two hypotheses in the present study. The first one was that the renal toxicity is maximal when gentamicin is given in the middle of the resting period, and minimal when administered in the middle of the activity period. The second one was that a difference in the temporal variations of food and water intakes is observed in rats treated with gentamicin.

These hypotheses were tested by injecting adult female Sprague-Dawley rats with gentamicin (80 mg/kg, i.p.) or with saline (NaCl 0.9%, i.p.) at one of two time points of the day, i.e., 1300 h or 0100 h. One specific objective of the present study was to assess if the nephrotoxicity induced by gentamicin was higher when the animals were injected at 1300 h than at 0100 h by measuring the urinary excretion of three renal enzymes (N-acetyl-ß-D-glucosaminidase, ß-galactosidase, γ-glutamyltrasferase), serum creatinine, blood urea nitrogen, the intracortical accumulation of gentamicin, and the incorporation of [^{3}H]-thymidine in the DNA of the renal cortex. A second objective was to record food and water intakes in order to assess if a change in the temporal pattern of intakes (24 h, 14 h light and 10 h dark phases, early, middle and late parts of the light and dark phases) occurred within each group (gentamicin 1300 h, saline 1300 h, gentamicin 0100 h, and saline 0100 h) over the 10-day experimental period.

Chapter 2

LITERATURE REVIEW

The literature review provides background information on aminoglycosides (AG) and biological rhythms, and summarizes what is currently known about the relationship of AG-induced nephrotoxicity and feeding. The three areas should essentially provide a thorough background for the present research.

1. THE AMINOGLYCOSIDES

1.1 Structure, Mode of Action, and Pharmacological Properties

The AG such as gentamicin, tobramycin, amikacin, streptomycin, netilmicin, kanamycin, neomycin, and isepamicin are a family of broad-spectrum antibiotics composed of a central hexose nucleus to which two or more amino sugars are bound by a glycosidic linkage (Randall et al., 1987). Being polycationic and highly polar, their absorption from the gastrointestinal tract is negligible and their crossing of the blood-brain barrier is poor, so that the concentrations reached in the cerebro-spinal fluid are inadequate for the treatment of infections (Randall et al., 1987; Sande and Mandell, 1990). Parenteral administration is necessary in order to obtain adequate serum concentrations for treatment of systemic infections (Randall et al., 1987).

Aminoglycosides penetrate the outer envelope and cytoplasmic membrane of bacteria by an oxygen-requiring and energy-dependent process; anaerobic organisms are therefore resistant to their action. Aminoglycosides lead to cell death by binding irreversibly to the 30S bacterial ribosome, thus causing a misreading of the genetic code and leading to a disruption in normal protein production (Randall et al., 1987; Sande and Mandell, 1990; Malseed et al., 1995).

The principal use of AG is against gram-negative bacilli such as *Pseudomonas, Klebsiella, Serratia, Escherichia, Enterobacter and Proteus* species. They are used for the

treatment of serious infections of the bone, skin, soft tissues, respiratory and urinary tracts, and for the treatment of peritonitis and sepsis (Association Pharmaceutique Canadienne, 1995; Deglin and Vallerand, 1995; Malseed et al., 1995).

Similar pharmacokinetic properties are shared by all AG. They are distributed in all extracellular liquids, and their binding to plasma albumin is not significant. Peak serum concentrations are rapidly attained: 30 to 90 minutes following an intramuscular (i.m.) injection and in the following 30 minutes of an intravenous (i.v.) infusion. Their serum half-life varies between 2 to 4 hours in human adults with normal renal function. Aminoglycosides are not metabolized systemically; they are excreted intact in the kidneys via glomerular filtration, and only 1% of the administered dose is eliminated in the bile. In the urine, their concentration can be as high as a hundred times the serum level (Randall et al., 1987). The renal clearance of these drugs is similar to the rate of creatinine clearance. The dose given to adult patients varies from 3 to 6 mg/kg of body weight (b.w.) per day, and must be calculated according to the renal function and blood levels (Randall et al., 1987; Deglin et al., 1995; Malseed et al., 1995; Sande and Mandell, 1990).

1.2 Nephrotoxicity

The major limitation to the routine use of all AG antibiotics is their potential nephrotoxicity. It can develop even with normal therapeutic doses, usually after a week or so of treatment (Randall et al., 1987; Malseed et al., 1995). Approximately 8 to 26% of hospitalized patients receiving these antibiotics will develop renal impairments (Kahlmeter and Dahlager, 1984). Because of the renal epithelium's regenerative ability, the toxic injury to the proximal tubule is generally a reversible process upon discontinuation of the drug regimen, but because of drug accumulation and tissue half-life of several hundred hours (Appel, 1990) full recovery may take several weeks (Mathew, 1992). The nephrotoxic potential of AG complicates the patients' conditions, prolongs the hospital stay, and increases medical costs (Sande and Mandell, 1990).

Following filtration through the glomerulus, a small portion of the AG binds to the brush-border membrane of the proximal tubular cells and is reabsorbed by adsorptive endocytosis, the predominant pathway of intracellular AG uptake (Silverblatt and Kuehn, 1979). The uptake at the basolateral level via tubular secretion is minor. The drug then accumulates in the lysosomes of the proximal tubular cells (Appel, 1990).The acidic phospholipids of the brush border membrane have been identified as the binding site for AG, especially the molecule phosphatidylinositol-4-5'-bisphosphate (Sastrasinh et al., 1982). Aminoglycosides are basic and cationic, whereas phospholipids are acidic and anionic, thus the binding is due to a charge interaction between the amino group of the AG and the phospho group of the acidic phospholipids (Sastrasinh et al., 1982; Mingeot-Leclercq et al., 1995).

There are two key elements explaining the susceptibility of the kidney to toxic damage induced by AG: the accumulation in the proximal tubular cells and the interaction with cellular membranes and organelles. The brush-border membrane cells of the proximal tubules are exposed to a concentration of AG greater than that found in the serum since the kidney is the major excretory route of the drug. The serum level required for clinical efficacy appears to be too low for the binding reaction to take place in other organs (Sastrasinh et al., 1982). Once inside the proximal tubular cells, the drug is in a poorly exchangeable pool. The reabsorption of AG and their long tissue half-life lead to toxic concentrations that can be attained even during standard therapy (Kaloyanides, 1991). Eventually, proximal tubular cell damage occurs.

Various potential membrane and metabolic disturbances leading to this damage have been hypothesized. The accumulation of undigested phospholipids at the lysosomal level, referred to as lysosomal phospholipidosis, appears to be closely related to the AG nephrorotoxicity. This phospholipidosis would be due to the inactivation of the lysosomal phospholipase activity caused by the binding of the AG to the acidic phospholipids of the lysosomal membrane (Laurent et al., 1982). As mentioned earlier,

the binding of AG to cellular membranes is electrostatic in nature as it is dependent on the pH and the ionic strength and it requires the presence of negatively charged phospholipids, which are present in 10 to 30% of most cellular membranes (Laurent et al., 1982). Lysosomes have a more acidic pH (around 5.5) than the cytosol and other organelles, and that may explain the preference of the AG for the lysosomal phospholipid bilayer. Other factors supporting the relationship between the lysosomal phospholipidosis and cellular necrosis is that modulation of the phospholipidosis also modulates the nephrotoxicity. Beauchamp et al.(1986, 1990) have demonstrated that poly-L-aspartic acids (PAA) can almost completely prevent the lysosomal phospholipidosis induced by gentamicin as reported by a suppression of the signs of nephrotoxicity. Kishore et al. (1990a, 1990b) showed that PAA prevented the phospholipidosis by binding with the AG in the lysosomes. It is not clear as to how the phospholipidosis leads to cell death. The leading hypothesis is that the drug and phospholipids accumulate up to a certain concentration at which point the lysosomal membrane breaks apart, resulting in the liberation of lysosomal hydrolases and large amounts of AG in the cytoplasm. Acute tubular necrosis develops when cellular regeneration can no longer compensate for focal necrosis (Tulkens, 1986; Kaloyanides, 1991). Mingeot-Leclerq et al.(1995) on the other hand, do not support this hypothesis of lysosomal rupture arguing that the destroyed lysosomes observed in the few in vivo reports could be artifactual in nature. Consequently, it appears that the lysosomal phospholipidosis and the AG nephrotoxicity are related but the exact mechanism leading to cell death deserves further attention.

The clinical manifestations of AG nephrotoxicity have been well studied in experimental animals and in humans. Enzymuria, or the excretion of brush-border and lysosomal enzymes in the urine, is the initial sign of damage. Polyuria also develops, possibly due to a decrease in the concentrating ability of the kidney and to resistance to vasopressin. Glycosuria, proteinuria, potassium and magnesium wasting, and the appearance of casts in the urine are also observed. A decline in glomerular filtration rate,

associated with a rise in blood urea nitrogen and plasma creatinine are the late external manifestations of renal damage (Humes, 1988; Appel, 1990; Sande and Mandell, 1990).

1.3 Risk and Protective Factors

Several variables have been identified in animal and clinical studies as potential risk factors in an attempt to reduce the possibility of developing renal problems in the course of AG treatment. They are summarized in Table 1 and classified as factors that the clinician may modify and factors that cannot be influenced readily by the clinician (Bertino et al. 1993; Humes 1988; Meyer 1986; Moore et al. 1984; Randall et al., 1987; Tulkens 1986; Whelton 1985). However, conflicting results were obtained for some of these risk factors and renders their impact uncertain. For example, female gender was identified as a risk factor in the clinical study of Meyer (1986) but this was not confirmed by other studies in humans (reviewed by Kahlmeter and Dahlager, 1984). Bertino et al. (1993) concluded that no risk factor, alone or in combination, have been found to be sensitive enough to predict an AG-associated nephrotoxicity reliably and this is in agreement with others (Lam et al., 1986).

Various protective approaches, listed in Table 2, have been proposed to ameliorate the nephrotoxicity side effect of AG. To this day, the once-a-day dosing approach is the only one currently used in clinical settings from all the approaches proposed from research done on animals in an attempt to find a method of reducing the incidence and severity of nephrotoxicity induced by AG (Beauchamp et al., 1995). It has been demonstrated in humans that reducing the injections to once a day rather than thrice a day was as effective, in addition to being more protective against renal toxic response (Prins et al., 1993, Nicolau et al., 1995). The frequency of injection is important as it mostly determines the serum and cortical concentrations of the antibiotics. High peak serum levels are reached with this regimen, and AG have a "post-antibiotic" effect so that bacterial killing rate can

be maximized. The fact that the uptake of AG by the renal cortex is a saturable process (Giuliano et al., 1986), and that there are prolonged drug-free interval with the single-daily dosing regimen, may contribute to the lower incidence of toxicity. The time of the day at which this single daily dose is administered is, however, still disregarded. This approach is discussed in this review under the heading Aminoglycosides Circadian and Seasonal Toxicity Variations (section 2.3). The effect of certain dietary factors to prevent experimental AG nephrotoxicity have been studied but their clinical application remains uncertain. In a recent review by Ali (1995), dietary calcium loading, pyridoxal-5-phosphate and fish oil supplementation were among treatments that proved to be protective against AG-induced nephrotoxicity.

TABLE 1

Proposed Risk Factors for Aminoglycoside Nephrotoxicity

Influenced by the Clinician
Large total dose
Long treatment duration
Hypokalemia
Hypoalbuminemia
Dehydration
Hypovolemia, hypotension, shock
Concurrent medications with nephrotoxic potentials
Not Influenced by the Clinician
Advanced age
Preexisting renal disease
Preexisting hepatic disease
Recent aminoglycoside therapy
Gender
Obesity

(adapted from Whelton, 1985)

TABLE 2

Proposed Protective Factors for Aminoglycoside Nephrotoxicity

Dosing intervals
Time of administration
Dietary factors

(adapted from Ali, 1995)

2. BIOLOGICAL RHYTHMS

2.1 Basic Concepts

It was once believed in biology and medicine that living organisms did not display temporal variations. In 1957, Reinberg and Ghata proposed to recognize rhythmicity as a fundamental characteristic of living organisms (Reinberg et al., 1989). Biological rhythms of 24 h (circadian) have been identified in most organisms, from single cell organisms to man, and in every function studied (Aschoff, 1980). Using a statistical and biological arguments, Halberg introduced the prefix circa (from Latin *circa*, about) to describe biological rhythms. The duration of a period is an estimation, and this duration can be modified by certain experimental conditions such as free-running conditions, under which an organism is kept in isolation and deprived of any periodic input. For these two reasons, the adjectives circadian, circaseptan, circannual, etc., are used to refer to endogenous rhythms, with a period of about 24 h, seven days, one year, etc. (Reinberg et al., 1991). A biological rhythm can be characterized and quantified by a periodic function with a maximum (acrophase) and a minimum (bathyphase) for a specified period (Reinberg et al., 1991).

The study of biological rhythms gives the means to answer the question "when?". Answering this question is an essential complement to the traditional questions of "where?" and "how?" asked in biology (Reinberg, 1989). Chronobiology is thus the science investigating the biologic time structure of organisms, its alterations and the mechanisms responsible for its control and maintenance (Halberg et al., 1977). Biological rhythms probably correspond to an adaptation of living organisms to the predictable cyclical oscillations of the environment. Organisms are therefore temporally adapted to the cyclic changes of the environment (Marques and Waterhouse, 1994). Periodic factors in the environment act as synchronizing agents, or Zeitgebers ("time-givers"). They entrain the endogenous self-sustaining rhythms and calibrate their period. The most powerful synchronizer of animals is the light-dark cycle. For humans, however, the social

surrounding with its activity-rest or sleep-wakefulness pattern, is the most important entraining agent. It is important to note that synchronizers do not generate rhythms (Reinberg et al., 1991). In fact, when every synchronizers are eliminated by placing organisms in a constant environment, the rhythms are maintained and display their natural period; they are said to "free-run" (Zucker, 1980).

Chronobiological observations have led to the development of the new scientific discipline of chronopharmacology, officially recognized by the scientific community in the early 1970s. Chronopharmacology is concerned with the activity, toxicity and kinetics of drugs according to their time of administration in relation to the synchronization of the organism, and investigates the possible alterations of the temporal structure of the organisms receiving the drug (Reinberg, 1989). Results from experimental and clinical studies following the chronopharmacologic methodology can be used for chronotherapy, that is optimizing therapy through the choice of the best-time of administration of a drug in order to enhance the desired side effects and decrease the undesired side-effects (Reinberg et al., 1991).

2.2 Feeding Rhythms

Circadian rhythms are displayed in food and water ingestions. The highly predictable alternance of day and night influences food availability and predator activity, thus resulting in animals being active either at night or during the day (Nagai et al., 1992). Food gathering and eating occurs when the animals are awake and active, therefore there are two classes of animals in terms of feeding: nocturnal animals, including rodents, eat at night, and diurnal animals, including humans, eat in the daytime. Rats generally consume more than 70% of their food and water during the dark phase (Zucker, 1971), and at least 70% of the rats water consumption occurs in close association with the meals (Fitzsimons and LeMagnen, 1969; Plaza et al., 1993). Rats display a bimodal distribution of feeding and drinking in the dark phase with a peak in the beginning and another peak towards the

end of this active period (Siegel, 1961). The food ingested during the first half of the dark phase has been shown to be used to supply immediate energy needs and promote lipogenesis (Le Magnen and Devos, 1970). In contrast, during the second half of the dark phase, food is used to replenish the nutrient and energy stores for their subsequent utilization during the light phase (Le Magnen and Devos, 1970; Armstrong et al., 1978).

2.3 Aminoglycosides Circadian and Seasonal Toxicity Variations

Experimental work in rodents has shown temporal variations in AG-induced toxicity. Nakano and Ogawa (1982) were the first experimenters to show a circadian variation in the mortality rate of male ICR mice adapted to a 12:12 light:dark cycle (lights on at 0700 h) following a single injection of gentamicin (285 mg/kg s.c.) at one of the following six time points: 0900 h, 1300 h, 1700 h, 2100 h, 0100 h and 0500 h. A peak of mortality was found when the animals were treated in the middle of the light phase (1300 h) and a trough toward the end of the dark phase (0500 h): significantly more animals died when injected during the light as opposed to the dark phase. This circadian rhythm in the susceptibility of mice to an acute dose of an AG was observed in two other lethality experiments. Several dose ranges of gentamicin (250, 275, 300 and 325 mg/kg i.m.), dibekacin (320, 355, 390, 425 and 460 mg/kg i.m.) and netilmicin (120, 130, 140 and 145 mg/kg i.m.) were injected at four different time points of the day (0800 h, 1400 h, 2000 h and 0200 h) to female Swiss mice maintained under a 12:12 light:dark cycle (lights on at 0800 h), and it appeared that the percentage of mortality was minimal when animals were treated during the dark period (2000 h, 0200 h) but maximal during the light period (0800 h, 1400 h) (Pariat et al., 1984). The latter experiment was performed between the months of February and September. Similar results were obtained when a single lethal injection of amikacin (1600, 1700, 1800 or 1900 mg/kg i.p.) was administered to female Swiss mice kept under a 12:12 light:dark cycle (lights on at 0800

h) at four times of the day (0800 h, 1400 h, 2000 h and 0200 h) in the months of March and April where maximal toxicity was observed with the injection at 1400 h and minimal at 0200 h (Dorian et al., 1985). However, when performed in November and December, the same experimental procedures yielded opposite results: maximal toxicity was observed at 0200 h and minimal at 1400 h (Dorian et al., 1985). These results suggest a circannual variation in AG toxicity but due to the nature of this study, it is limited by the fact that the animals used in the Spring were not the same as the ones used in the Fall. The three above studies were the first to show that the time of day, and possibly the time of year, at which AG are administered are important factors to consider in toxicological studies. Due to the high doses used in these experiments, the death of the animals occurred about thirty minutes after the injection; the cause therefore cannot be attributed to dramatic renal damages but rather to a neuromuscular blockade (Nakano and Ogawa, 1982). The study of circadian variations in the nephrotoxicity of AG requires, therefore, the use of sublethal doses.

In a study, male Wistar rats synchronized to a 12:12 light:dark cycle (lights on at 0800 h) were treated with a high single sublethal dose of amikacin (1200 mg/kg i.p.) at four different time points of the day (0800 h, 1400 h, 2000 h or 0200 h) in October and in April (Dorian et al., 1986). The nephrotoxicity, assessed by the urinary excretion of γ-glutamyltransferase (γ-GT) in the urine 24 h following the injection, was maximal in the middle of the light phase (1400 h) in April and at the beginning of the dark phase (2000 h) in October. The results supporting the circannual variation in amikacin toxicity concur with those obtained in the acute toxicity study mentioned previously (Dorian et al., 1985). This assumption, however, is limited by the fact that only one enzyme was used to reach this conclusion. Dorian et al. (1987) also observed a maximal damage to the kidneys in male Wistar rats adapted to a 12:12 light:dark cycle after 7 daily injections of amikacin (400 mg/kg i.m.) when the drug was given at 1400 h compared to injections at 0800 h, 2000 h or 0200 h in March and April. Time-dependent nephrotoxic effects of AG were

also reported in a study assessing parameters of renal function. Indeed, following a single i.p. injection of amikacin (1200 mg/kg b.w.) at one of four time points (0400 h, 1000 h, 1600 h or 2200 h) in male Wistar rats adapted to a 12:12 light:dark cycle (lights on at 0700 h), a greater decrease in creatinine clearance and tubular function was found when the drug was administered during the light phase (1600 h) than during the dark phase (Fujimura et al., 1994).

Circadian variations in the nephrotoxicity of AG were also reported with the use of low doses. Renal toxicity, assessed by the activity of N-acetyl-ß-D-glucosaminidase (NAG), histopathological observations and plasma AG level, was more severe in 7 weeks old male Wistar rats adapted to a 12:12 light:dark cycle (lights on at 0700 h) and injected for 8 consecutive days with gentamicin (60 mg/kg s.c.) at midlight (1300 h) than in rats injected at middark (0100 h) (Yoshiyama et al., 1992). Lin et al. (1994) reported that following 4 days of treatment with tobramycin (40 mg/kg, i.p.) in female Sprague-Dawley rats adapted to a 14:10 light:dark cycle (lights on at 0600 h), no significant difference was observed in the circadian variation of the nephrotoxicity among the groups injected at 0800 h, 1400 h, 2000 h or 0200 h. After 10 days of treatment, however, the cellular regeneration, a sensitive marker of renal toxicity, was the highest in the group treated at 1400 h and the lowest in the group treated at 0200 h. This experiment was repeated in the Spring, Summer, Fall and Winter, and no circannual variation were observed in the toxicity. The same group of researchers also demonstrated similar results with single daily injection of isepamicin (80 mg/kg i.p.) or saline given for 10 days to adult male Sprague-Dawley rats kept under a 14:10 light:dark cycle (lights on at 0600 h). The animals were treated at 0800 h, 1400 h, 2000 h or 0200 h, and it was shown that renal toxicity was maximal when isepamicin was given at 1400 h (midlight) and minimal at 0200 h (middark), as demonstrated by significantly higher levels of cellular regeneration and isepamicin cortical accumulation in animals treated at 1400 h than in those treated at 0200 h (Yoshiyama et al., 1996a).

The above mentioned studies fully support the existence of temporal variations in AG-induced nephrotoxicity, since mice and rats of different strains and of both sexes were used, different doses of various AG were administered for variable number of days, and different parameters of toxicity were measured.

Few human data are available on the circadian variations in pharmacokinetics and renal toxicity of AG. The influence of time of day of drug administration (0800 h and 2000 h) on the pharmacokinetics of an i.m. injection of 400 mg of isepamicin given to six healthy subjects (three adult men, three adult women) was studied by Yoshiyama et al. (1996b) in a randomized cross-over design with one week washout. The subjects maintained a regular lifestyle, with sleep time from 2300 h to 0700 h. Blood samples were drawn at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8 and 12 hours after isepamicin administration and no difference was found between the morning and evening trials for the plasma peak concentrations, indicating that the clinical efficacy, which is dependent on blood concentrations, may not be different whether the drug is given in the morning or in the evening. However, a significant lower clearance and higher area under the time-concentration curve (AUC) were observed when isepamicin was administered at 2000 h when renal function is known to be lower in day-active individuals.

Prins et al. (1997) were the first experimenters to report evidence of temporal variation in the nephrotoxicity of AG in humans. Two hundred and twenty-one patients with severe infections were enrolled in a prospective study and treated once daily with gentamicin or tobramycin (standard dosage: 4 mg/kg i.v.). The time of first dosing became the time of administration of all subsequent dosages and three time periods were studied (0000 h to 0730 h [resting period], 0800 h to 1530 h, and 1600 h to 2330 h). Serum trough and peak drug levels were not significantly different among the three time periods, but nephrotoxicity, assessed by a rise in serum creatinine, occurred significantly more often when the AG were given between 0000 h and 0730 h (resting period) compared to the other two periods.

3. AMINOGLYCOSIDES TOXICITY AND FEEDING

Changes in the susceptibility of renal cells according to the time of day, circadian variations of endogenous hormones secretion and changes in the serum and intracortical pharmacokinetics of AG are among the mechanisms proposed to explain the temporal variation in the nephrotoxicity, or chrononephrotoxicity, of this family of drugs (Beauchamp et al, 1995; Lin et al, 1994). Another recently proposed possible mechanism is the rhythmic pattern of food and water ingestions. As mentioned in section 2.5, the minimal toxicity in rodents is consistently observed during the dark (activity) period, which corresponds to the maximal food and water intake period of this nocturnal species.

3.1 Food deprivation

The first investigation on the effect of a short period of food deprivation on time-dependent variations in gentamicin nephrotoxicity was conducted in 1996 by Beauchamp et al. In July, adult female Sprague-Dawley rats adapted to a 14:10 light:dark cycle (lights on at 0600 h) were offered food and water ad libitum or were fasted 12 h before and 24 h after (water was available ad libitum) a single injection of saline (NaCl 0.9%, i.p.) or of gentamicin (150 mg/kg i.p.) given at 1400 h or at 0200 h. In the group fed ad libitum, the excretion of urinary enzymes, the cellular regeneration and the level of gentamicin renal cortical accumulation were significantly higher when gentamicin was given at 1400 h than at 0200 h. Compared to the time-matched saline-treated animals, rats treated at 1400 h had significantly higher values for these nephrotoxicity parameters, but in the animals injected at 0200 h there was no difference between the ones treated with gentamicin compared to the ones treated with saline, therefore concurring with previous studies showing a peak and a trough of toxicity at these time points. In the fasted animals, however, there was no difference between the groups injected at 1400 h and 0200 h, both groups had significantly higher values for the nephrotoxicity parameters compared to their respective controls. The peak and trough of nephrotoxicity were abolished by food deprivation. The

authors suggested that fasting could have increased gentamicin nephrotoxicity by lowering the clearance of the drug and by possibly inducing a shift in the acrophase and bathyphase of nephrotoxicity. This shift, however, could not be detected since only two time points of the day were examined in the study.

Using a similar fasting/feeding design, the same group of researchers examined the effects of fasting on the temporal variations in nephrotoxicity and in the serum and intrarenal distribution of tobramycin in female Sprague-Dawley rats adapted to a 14:10 light:dark cycle (lights on at 0600 h) in the Spring (Lin et al., 1996). In a toxicity study, animals were injected with a single dose of tobramycin (150 mg/kg i.p.) at 1400 h or 0200 h, and it was found that the levels of ß-galactosidase (ßGal) in the urine were significantly higher in fasted rats treated at 0200 h than in fed rats treated at the same time of day. This effect was not observed in the fed and fasted animals treated at 1400 h. Serum and renal cortical tobramycin levels were higher in fasted rats injected at 1400 h than at 0200 h, and than in fed rats injected at 1400 h. These results suggested an increase susceptibility of the kidney to tobramycin-induced toxicity in the fasted state. For the serum and renal distribution study, the animals were treated with a single injection of tobramycin (40 mg/kg i.p.) and then sacrificed 1, 2, or 4 h following the injection. In groups treated at 0200 h, the serum and cortical levels were significantly higher in the fasted animals compared to the fed animals after 2 h and 4 h following the injection. When the drug was given at 1400 h, the serum and cortical levels did not differ between the fasted and fed rats.

Further research should be done to determine whether fasting alters the chrononephrotoxicity of AG in rats by inducing a shift in the acrophase and bathyphase of toxicity. These results, however, suggest that the presence or absence of foods has a significant influence on the temporal variations of the distribution and renal toxicity of AG.

3.2 Time-restricted feeding schedules

Song et al. (1993) examined the influence of manipulating feeding schedules on gentamicin toxicity rhythms. Between the months of November and January, ICR male mice housed in a room equipped with a 12:12 light:dark cycle (lights on at 0700 h) were adapted for 14 days to an ad libitum feeding schedule (ALF) or to a time-restricted feeding schedule (TRF) where food was made available at a time when food consumption is normally minimal (feeding time: 0900 h to 1700 h). The animals were injected daily either at midlight (1300 h) or at middark (0100 h) with gentamicin (180 or 220 mg/kg s.c.) for 18 days. Body weight loss was used as the end point for the lower dose and mortality for the higher one. In the ALF group, a significant dose-time dependency was observed as body weight loss was more rapid and mortality higher among mice treated at 1300 h compared to the ones treated at 0100 h. Opposite results were observed in the TRF group: the toxicity was lower when gentamicin was given at 1300 h (food available) and higher at 0100 h (food not available). The chronopharmacokinetics of gentamicin was studied in another experiment, where one group was adapted to the ALF schedule for 14 days, a second group to the TRF schedule. The animals were injected with gentamicin (180 mg/kg s.c.) at either 0900 h, 1300 h, 1700 h, 2100 h, 0100 h, or 0500 h and multiple blood samples from the orbital sinus were drawn 0.5 h, 1 h, 2 h, 3 h, and 4 h following each treatment. Under the ALF schedule, the mean plasma drug concentrations at 0.5 h and 1 h and the AUC were significantly higher and the total body clearance was lower in mice injected at midlight, whereas under the TRF schedule the kinetic rhythm was shifted by about 12 h. These results indicate that under the ALF and the TRF conditions the maximal toxicity coincided with the higher plasma drug concentration and lower clearance of the drug. The authors suggested that under TRF conditions, the drive for food dominates and probably entrains a change in the animal's activity and sleep-wake pattern.

Beauchamp et al. (1997) examined gentamicin-induced nephrotoxicity relative to food access periods. Female Sprague-Dawley rats fully adapted to a 14:10 light:dark cycle

(lights on at 0600 h) and to 8 h feeding schedules (0800 h-1600 h, 1600 h-0000 h, or 0000 h-0800 h) were treated with gentamicin (40 mg/kg i.p.) for 4 and 10 days at 0700 h, 1300 h, 1900 h, or 0100 h. After 10 days of treatment, the maximal toxicity assessed by changes in the inhibition of sphingomyelinase activity, cellular regeneration, blood urea nitrogen, serum creatinine levels and histopathological lesions was always obtained when the injections were given when food was not available. Time-restricted feeding schedules moved the peak and trough of nephrotoxicity. It was also demonstrated by a multivariate ANOVA that feeding state (fed, intermediary or fasted) was more important than the activity status (active or rested) on gentamicin nephrotoxicity. The authors concluded that the time of injection relative to food availability was more important in gentamicin-induced nephrotoxicity modulation than the light-dark cycle.

In summary, it is clear that temporal variations in AG-induced nephrotoxicity exist, with a maximum nephrotoxicity observed when the drug is administered in the middle of the animal's resting period and a minimum nephrotoxicity when injected in the middle of the animal's activity period. This temporal variation could partly be explained by the increased renal function, shorter elimination of the drug, and lower AUC observed during the activity period, and by changes in the susceptibility of renal cells according to the time of day. Another recently proposed mechanism is the rhythmic pattern of food and water intakes. A short fasting period and a time-restricted feeding schedule modify the variations in nephrotoxicity normally observed in ad libitum fed rats. Minimal renal toxicity is consistently observed when the injection is given when food is available. No studies to this day have measured food and water intakes during an AG treatment in rats fed ad libitum.

Chapter 3

MANUSCRIPT

Temporal Modulation of Nephrotoxicity and of Feeding and Drinking by Gentamicin Treatment in Rats

Temporal Modulation of Nephrotoxicity and of Feeding and Drinking by Gentamicin Treatment in Rats

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ABSTRACT

JULIEN, N., M. KARZAZI, G. LABRECQUE, D. BEAUCHAMP AND L. THIBAULT. Temporal modulation of nephrotoxicity and of feeding and drinking by gentamicin treatment in rats. PHYSIOL BEHAV XX(X) 000-000, 199X.— Gentamicin-induced nephrotoxicity varies temporally, with a peak being observed when this antibiotic is administered during the resting period and a trough when given during the activity period of rats. These variations are modified by fasting and by restricted feeding schedules. In this study, food and water intakes of adult female Sprague-Dawley rats were measured during pre-treatment (days 1 to 5) and during treatment (days 6 to 10) with gentamicin (80 mg/kg/day, i.p.) injected at 1300 h or 0100 h. A significantly higher level of serum creatinine was observed when gentamicin was administered at 1300 h compared to 0100 h, and a significantly lower creatinine clearance was found in rats treated with gentamicin at 1300 h compared to those treated with saline at the same time. Gentamicin treatment at 1300 h or 0100 h resulted in a decrease in the 24 h food intake. In addition, in the gentamicin-treated group at 0100 h, the maximal food intake observed at late dark during the pre-treatment period decreased during treatment, and early dark rather than late dark maximal intake occurred. Our data demonstrate that gentamicin induces a nephrotoxicity that varies temporally, and that gentamicin treatment inhibits food intake and alters its nocturnal variations.

Gentamicin Nephrotoxicity Temporal Variation Feeding Drinking Rats

INTRODUCTION

Aminoglycosides (AG) are large spectrum antibiotics that have been used in hospital settings for many years, alone or in combination with other antibiotics, to combat severe gram-negative bacterial infections. Their efficacy, however, is shadowed by the fact that all AG have the potential to induce renal toxicity as a side effect (Sande and Mandell, 1990). This nephrotoxicity occurs in 8% to 26% of hospitalized patients receiving these antibiotics (Kahlmeter and Dahlager, 1984) and although it is generally reversible upon drug discontinuation it complicates the patients' conditions, prolongs the hospital stay, and increases the medical costs (Sande and Mandell, 1990).

Numerous studies performed in rodents have shown the renal toxicity induced by AG to vary temporally, with a peak observed when the treatment is given during the resting period of the animals and a trough when given during the activity period (Yoshiyama et al., 1992; Lin et al., 1994; Beauchamp et al., 1996; Lin et al., 1996; Yoshiyama et al., 1996a). Diurnal rhythms in food and water intakes are displayed in rodents, with intakes occurring mainly during their active period, the period that also corresponds to the trough of AG-induced nephrotoxicity. Previous work demonstrated the role the presence of food plays in modulating these temporal variations in rats exposed to a short fasting period (Beauchamp et al., 1996) or to a time-restricted feeding schedule (Beauchamp et al., 1997). When adult female Sprague-Dawley rats were fasted for 12 h before and 24 h after a single injection of gentamicin (150 mg/kg, i.p.), no temporal variations in nephrotoxicity were observed: the toxicity was maximal whether the animals were injected in the middle of their active or resting periods (Beauchamp et al., 1996). When adult female Sprague-Dawley rats were fed on 8-h feeding schedules and administered gentamicin (40 mg/kg/day, i.p.) for ten days, a minimal nephrotoxicity was constantly observed when they were treated during the period of food availability. The

time of injection relative to food availability was more powerful at modulating gentamicin-induced nephrotoxicity than the light-dark cycle (Beauchamp et al., 1997).

None of these studies, however, measured food and water intakes of animals. The purpose of this study, therefore, was to investigate the effect of gentamicin, given at times corresponding to the peak and trough of nephrotoxicity, on food and water intake patterns of rats fed ad libitum.

MATERIALS AND METHODS

Animals and Diets

Thirty-six adult female Sprague-Dawley rats (Charles River Breeding Laboratory, Montreal, Quebec, Canada), initially weighing 215-225g were used. The animals were housed in individual cages, in a room with controlled temperature and humidity and equipped with an automatic 14:10 light:dark cycle (lights on at 0700 h). This light/dark cycle was chosen to allow comparison with previous studies performed by our research group. During the 10-day experimental period, the animals were provided ad libitum with spring water (Nora Beverages Inc., Mirabel, Quebec, Canada) and commercial non-purified stock diet (18.1% protein, 4.5% lipid, 57.3% carbohydrate) reduced in a granular form (rat laboratory chow 5075, Charles River Breeding Laboratories, Montreal, Quebec, Canada).

Experimental Design

After 3 days of acclimatization to the environmental conditions, during which spring water and pelleted commercial non-purified diet were available ad libitum, the animals were adapted to the powder diet for 14 days before the beginning of the study. The animals were randomly divided into two groups (n=18); one group scheduled to be treated at 1300 h and the other group at 0100 h. These times have been demonstrated to correspond to the peak and trough of gentamicin-induced nephrotoxicity in our laboratory (Lin et al., 1994). These groups were further subdivided into two subgroups (n=9) in order to form the control and treatment groups. The animals were then placed in a Diet Scan data acquisition system (AccuScan Instruments Inc., Columbus, Ohio, USA) and were studied over an experimental period of 10 days. Measurement intervals of 1 minute across 24 h were used. Recordings of days 1 to 5 were used to characterize the rhythmicity of food and water intakes under normal conditions (pre-treatment period) and recordings of days 6 to 10 were used to characterize their rhythmicity under a pharmacological treatment (treatment period). Rats were administered gentamicin (kindly

provided by Schering Canada Inc., Pointe-Claire, Quebec, Canada) daily (days 6 to 10) at a dose of 80 mg/kg given intraperitoneally (i.p.) or an equivalent volume of saline (NaCl 0.9%). Based on the results of a pilot study (Beauchamp et al., unpublished results), 80 mg/kg was selected as the dose capable of inducing nephrotoxicity after five days of treatment. Body weight was measured daily. The maintenance sessions (food cups and water bottles refilling, body weight measurements, cleaning of cages and resetting of the Diet Scan system) were scheduled at 0900 h. Twenty-four to thirty-six hours following the last injection the animals were sacrificed by decapitation. Exactly one hour prior to their sacrifice each rat was injected with a single dose of [^{3}H]-thymidine (200 µCi: Amersham Canada, Oakville, Ontario, Canada) to measure cellular regeneration. Blood was collected immediately, centrifuged at 4°C and the serum was frozen at -80°C for future analysis. A central medial abdominal incision was done and the kidneys were removed and dissected. The renal cortex was dissected, put on dry ice immediately and stored at -80°C for future analysis. The experiment was conducted in the Summer.

Diet Scan System

The Diet Scan system (AccuScan Instruments Inc., Columbus, Ohio, USA) is an automated computer data acquisition system designed to record food and water intakes and to study food and water ingestion patterns of small animals by a continuous recording. Each Diet Scan cage was made of clear acrylic frames (41.75 X 41.75 X 31.5 cm^3) and was divided in two by a diagonally inserted acrylic frame so two animals were individually housed in one cage. The cages were equipped with four electronic balances (Ohaus Port-O-Gram-C301P and A&D-EW300A). The scales were connected to an analyzer which in turn was connected to an IBM PC computer programmed to record each scale's display every minute across 24 h. Each animal had access to a square opening in the cage giving it free access to a diet box placed on top of the scale and filled with the diet. Access to water was through a drinking spout connected to a drinking bottle by a sipper tube; the bottle was held by a holder on the scale plate. The data was collected using Diet Scan Lab

Animal Monitoring System Software (DLAM). A Diet Scan custom made template was used to compile the information into data files.

Biochemical analysis

The extent of cellular regeneration was evaluated by the incorporation of [^{3}H]-thymidine into the DNA of the renal cortex by the method described by Laurent et al. (1983). Serum and urine creatinine levels and blood urea nitrogen were determined by an automated enzymatic method using an Hitachi 737 analyzer. Creatinine clearance was calculated by multiplying urine creatinine concentration by urine volume and dividing this product by the serum creatinine concentration.

Enzymuria

All animals were placed individually in metabolic cages on the last day of adaptation to the diets and urine samples were collected in mineral oil for 24 h to obtain baseline values prior to the experimental period. Subsequent urine samples were collected for the 24 h following the last injection. The volumes were noted, the urines were immediately centrifuged, and the enzyme activities were measured within the two hours following urine collection. The determination of gamma-glutamyltransferase (γ-GT) excretion was performed using the method developed by Persijn and van der Slik (1976). The determination of ß-galactosidase (ßGal) and N-acetyl-ß-D-glucosaminidase (NAG) excretions were performed using the colorimetric method of Maruhn (1976).

Gentamicin cortical levels

The cortical accumulation of gentamicin was determined using a fluorescence polarized immunoassay (TDX System: Abbott Diagnostics, Mississauga, Ontario, Canada). Cortical renal samples were homogenized in cold distilled water (4°C) using a Tissue-Tearor RTM (Biospec Products, Bartleville, OK, USA). The homogenates were then sonicated (sonicator model W-375, Bionetics Ltd., Montreal, Quebec, Canada) for 15 seconds and diluted in TDX buffer. The sensitivity of the assay was 3 µg/g. The coefficients of variation were 3.44 % at 1.0 µg/ml and 2.72 % at 8.0 µg/ml.

Statistical Analysis

The nephrotoxicity parameters were analyzed by a two-way ANOVA using treatment (gentamicin vs. saline) and time of day at which the injection was given (1300 h vs. 0100 h) as main effects; the interaction between treatment and time of day was also tested.

Daily food and water intakes and body weight gain throughout the experimental period (days 2 to 10) were analyzed in each group separately; the groups were separated on the basis of treatment and time of day at which the treatment was given, i.e., gentamicin 1300 h, saline 1300 h, gentamicin 0100 h, and saline 0100 h. Due to a recording problem with the Diet Scan apparatus, food and water intake data on day 1 of the experimental period had to be taken out of the analysis. Body weight gain and the 24 h food and water intakes were analyzed by a one-way repeated measures ANOVA with day (2 to 10) as the factor. Ingestion of food and water was analyzed by using phase (14 h light and 10 h dark; early, middle and late light and dark) and day (2 to 10) as main effects; the interaction between phase and day was also tested.

A separate two-way repeated measures ANOVA was conducted on data during the treatment period (days 6 to 10) in order to test for differences in food and water intakes due to treatment (gentamicin and saline) and day (6 to 10); the interaction between treatment and day was also tested. Groups were separated on the basis of time of day at which the treatments were given, i.e., 1300 h and 0100 h.

Significant main effects were tested using Tukey's test for multiple comparisons. Results are presented as means ± standard error of the mean (SEM). A probability of less than 5% was considered significant. Statistical analysis was performed using SuperANOVA Software version 1.11 (Abacus Concepts Inc., Berkeley, California, USA).

RESULTS

NEPHROTOXICITY PARAMETERS

Cellular Regeneration

For cellular regeneration, a significant overall main effect of treatment (gentamicin vs. saline) was found, $F(1, 29) = 21.75$, $p<0.0001$; the gentamicin group had significantly more [^{3}H]-thymidine incorporated into the DNA of their renal cortical cells. The groups treated with gentamicin had significantly higher levels than the groups treated with saline at 1300 h and 0100 h (TABLE A1; FIG. A1a).

Renal Function

For the level of serum creatinine, the main effect of treatment was significant, $F(1, 32) = 6.48$, $p<0.05$. The animals treated with gentamicin had significantly higher serum creatinine levels than those treated with saline. The main effect of injection time (1300 h vs. 0100 h) was also significant, $F(1, 32) = 5.37$, $p<0.05$, with the rats treated at 1300h having significantly higher serum creatinine levels than rats treated at 0100 h (TABLE A1). Within the group treated at 1300 h, serum creatinine was significantly higher in rats treated with gentamicin (FIG. 1a).

No significant main effects of treatment or injection time was found for the level of blood urea nitrogen (TABLE A1; FIG. A1b).

For creatinine clearance, a significant interaction between treatment and injection time was found, $F(1, 32) = 4.15$, $p<0.05$ (TABLE A1). The difference between the creatinine clearance rates of rats treated with saline and gentamicin at 1300 h was significantly greater than the difference between creatinine clearance rates of rats treated with saline and gentamicin at 0100 h. Gentamicin treatment at 1300 h resulted in a significantly lower creatinine clearance rate compared to the saline treatment given at the same time. However, no such difference was found in the 0100 h-treated groups (FIG. 1b).

A significant overall main effect of treatment was found for the diuresis, $F(1,32) = 5.03$, $p<0.05$. The increase in the urine output from baseline was significantly higher in rats treated with gentamicin than with saline (TABLE A1; FIG. A1c).

Enzymuria

A significant overall main effect of treatment was found for the excretion of ßGal, $F(1, 31) = 12.49$, $p<0.01$, NAG, $F(1, 31) = 10.81$, $p<0.01$, and for γ-GT, $F(1, 30) = 11.67$, $p<0.01$. The increase in the excretion of those three enzymes from baseline in the 24 h urine of rats treated with gentamicin was significantly higher compared to the rats treated with saline. The time at which the animals were treated did not have a significant effect. Within both injection times (1300 h, 0100 h), the enzymuria was higher for the animals treated with gentamicin than with saline (TABLE A1; FIG. A1d, e, f).

Gentamicin Cortical Levels

Injection time had no significant effect on the accumulation of gentamicin in the renal cortex (TABLE A1; FIG. A1g).

BODY WEIGHT

Daily variations in body weight gain (g/day) of animals are presented in FIG. 2. Body weight gain was significantly affected by day in the group treated with gentamicin at 1300 h $F(8,64)=2.696$, $p<0.05$, where the increase in body weight was more pronounced during the pre-treatment period than during the treatment period. In this group, from days 1 to 5, the average weight gain was of 3.7 ± 1.2 g/day in comparison to 0.1 ± 0.8 g/day from days 6 to 10. In the other experimental groups, no such statistical significant variations were found (TABLE A2).

FOOD INTAKE

24 h, and 10 h dark and 14 h light phases

Throughout the experimental period (days 2 to 10), the 24 h food intake was significantly affected by day in the groups treated with gentamicin at 1300 h, $F(8,56)=8.69$, $p<0.01$, and 0100 h, $F(8,64)=7.77$, $p<0.001$ (TABLE A3; FIG. 3). More specifically, a decrease in the overall 24 h intake during the treatment period (days 6 to 10) compared to the before treatment period (days 2 to 5) was observed. The 24 h intake analysis during the treatment period (days 6 to 10) revealed a significant interaction between day and treatment in groups injected at 1300 h, $F(4,60)=7.32$, $p<0.001$, and in groups treated at 0100 h, $F(4, 64)=3.67$, $p<0.05$. In both groups, 24 h food intake of saline-treated animals was higher than that of gentamicin-treated rats, and this was more pronounced towards the end of the treatment period (TABLE A4; FIG. A4a, b).

The diurnal phase (14 h light vs. 10 h dark) also had a significant effect on food intake in the groups treated with gentamicin at 1300 h, $F(1,12)=40.13$, $p<0.0001$, and at 0100 h, $F(1,10)=7.69$, $p<0.05$, with rats in both groups eating more during the dark phase than the light phase throughout the experimental period. In the groups treated with saline, however, a significant interaction between diurnal phase and day (days 2 to 10) was observed for the group treated at 1300 h and 0100 h ($F(8,64)=3.42$, $p<0.05$, $F(8,12)=10.52$, $p<0.0001$, respectively), with the animals increasing their diurnal intakes but decreasing their nocturnal intakes during treatment (days 6 to 10) when compared to before treatment (days 2 to 5) (TABLE A5; FIG. A5a, b). During the treatment period (days 6 to 10), the interaction between day and treatment was significant for the 10 h dark food intake in the groups treated at 1300 h, $F(4, 52)=8.73$, $p<0.001$. For the first three days of treatment (days 6 to 8) nocturnal food intake did not differ significantly between gentamicin- and saline-treated animals at 1300 h. On days 9 and 10, however, saline-treated had a higher food intake than gentamicin-treated (TABLE A6; FIG. A6a, b).

Early, middle and late parts of the dark and light phases

Throughout the experimental period (days 2 to 10), a significant interaction between parts of the dark phase and day was observed for the groups injected at 0100 h with gentamicin, $F(16,192)=3.76$, $p<0.001$ and saline, $F(16,168)=2,18$, $p<0.05$ (FIG. 4). In both groups before treatment (days 2 to 5), food intake was maximal at late dark, followed by early and middle dark respectively. During the treatment period (days 6 to 10) for the gentamicin-treated group, this pattern was reversed: the maximum intake was observed at early dark and minimal intakes at middle and late dark. In the saline-treated group, on the first three days of treatment, similar food intakes were observed at early, middle and late dark, whereas the intake at early dark was higher than at middle and late dark on the last two days of treatment (TABLE A8; FIG. A8a, b).

As shown in FIG. 5, food intake during the light phase in the group injected at 0100 h with gentamicin, was significantly affected by the parts of the light phase; the intake at middle light was significantly lower than at early and late light throughout the experimental period, $F(2,24)=15.90$, $p<0.0001$. For the group treated with saline at 0100 h, a significant interaction between phase and day was found, $F(16,160)=4.26$, $p<0.0001$. Before treatment, the maximal food intake was observed at early light and late light, and minimal at middle light. During the treatment period, however, the intake at early light was higher than at late light, and the minimal intake was still observed at middle light. For the gentamicin and saline groups injected at 1300 h, a significant interaction between parts of the light phase and treatment was found ($F(16,168)=4.79$, $p<0.0005$; $F(16,152)=5.71$, $p<0.01$, respectively) (TABLE A9; FIG. A9a, b).

WATER INTAKE

24 h, and 10 h dark and 14 h light phases

In the four experimental groups, the 24 h water intake was not significantly affected by day throughout the experimental period (days 2 to 10) (TABLE A10; FIG. A10a). During the treatment period (days 6 to 10), there was no significant difference between the gentamicin- and saline-treated groups for the 24 h water intake (TABLE A11; FIG. A11a, b).

The diurnal phase (14 h light vs. 10 h dark) had a significant effect on water intake in groups treated with gentamicin at 0100 h, $F(1,8)=43.16$, $p<0.0001$, and with saline at 1300 h, $F(1,12)=101.80$, $p<0.0001$, with rats drinking more during the dark phase than the light phase throughout the experimental period (days 2 to 10). In the groups treated with gentamicin at 1300 h or with saline at 0100 h, a significant interaction between diurnal phase and day was observed ($F(8,96)=2.25$, $p<0.05$; $F(8,80)=2.73$, $p<0.05$, respectively) (TABLE A12; FIG. A12a, b). During the treatment period (days 6 to 10), treatment did not have a significant effect on water intake during the dark and light phases in the four experimental groups (TABLES A13, A14; FIG. A13a, b; A14a, b).

Early, middle and late parts of the dark and light phases

Throughout the experimental period, the parts of the dark phase had a significant effect on water consumption of the saline-treated group at 0100 h, $F(2,15)=7.40$, $p<0.01$, with the intake being higher at early dark vs. middle and late dark. In addition, a significant interaction in the group injected with gentamicin at 0100 h was found between parts of the dark phase and day $F(16,144)=3.18$, $p<0.01$. Specifically, before treatment, the highest water intakes were in the late and early parts of the dark phase, followed by the middle part. During treatment, the intake at early dark was maximal, followed by the intakes at late and middle dark (days 6 and 7), middle and late dark (days 8 to 10) (TABLE A15; FIG. A15a, b).

A similar pattern was observed for the light phase water intake in the groups treated with gentamicin or saline at 1300 h, where a significant interaction between parts of the light phase and day was found ($F(16,144)=6.16$, $p<0.0001$ and $F(16,144)=3.09$, $p<0.01$, respectively). Before treatment (days 2 to 5), the lowest water intake was found in the middle part of the light phase in both groups compared to early and late light. During the treatment period (days 6 to 10), an augmentation in water intake at middle light occurred in the group treated with gentamicin, resulting in the highest intake being observed during that period. In the saline group, this increase at middle light was also observed during the treatment period, but it was not as pronounced as in the gentamicin-treated group (TABLE A16; FIG. A16a, b).

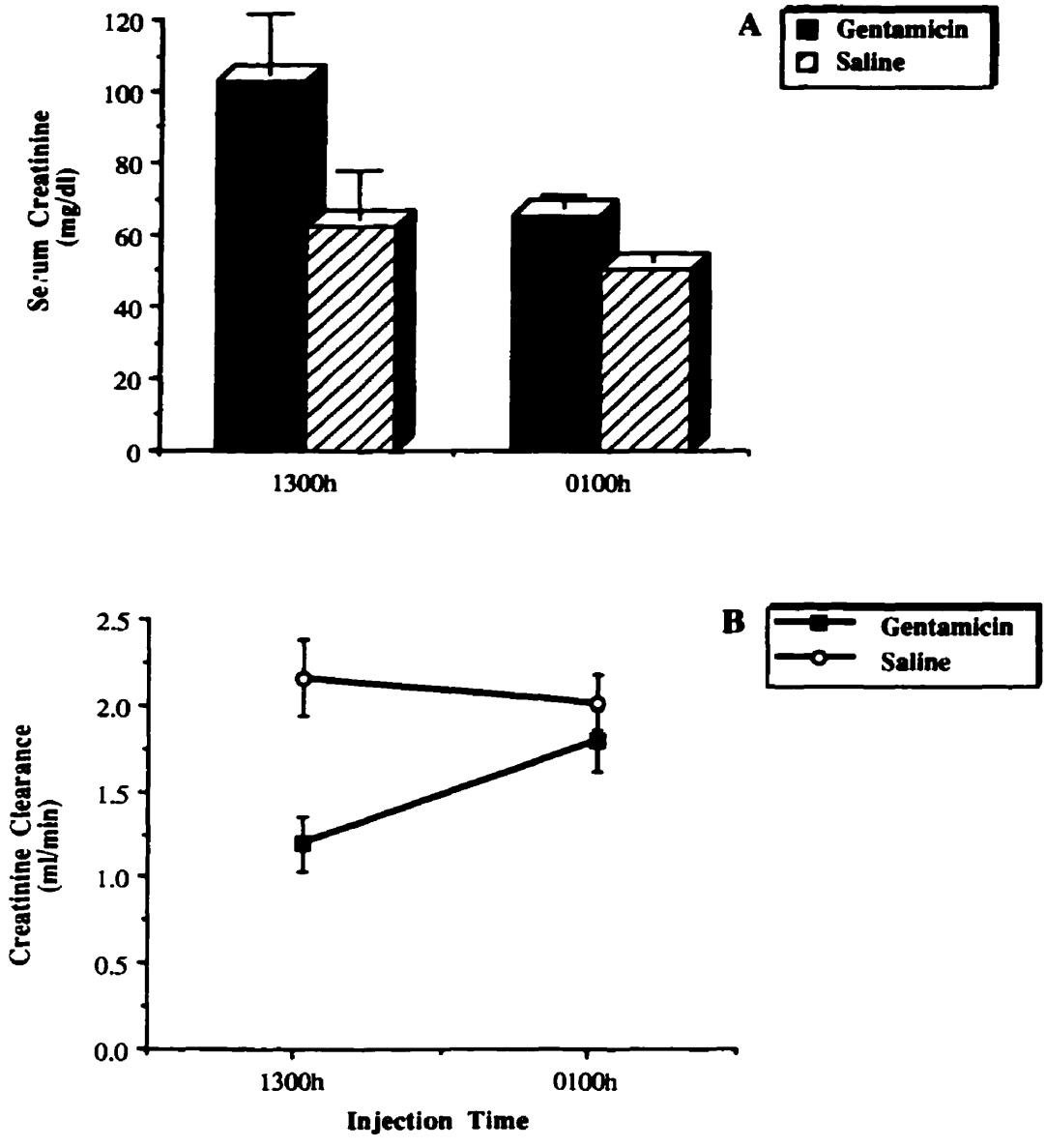


FIG. 1. Serum creatinine levels (A) and creatinine clearance rate (B) following five daily injections of gentamicin (80 mg/kg, i.p.) or saline given at 1300 h or 0100 h to rats. Data expressed as mean ± SEM. See text for significance.

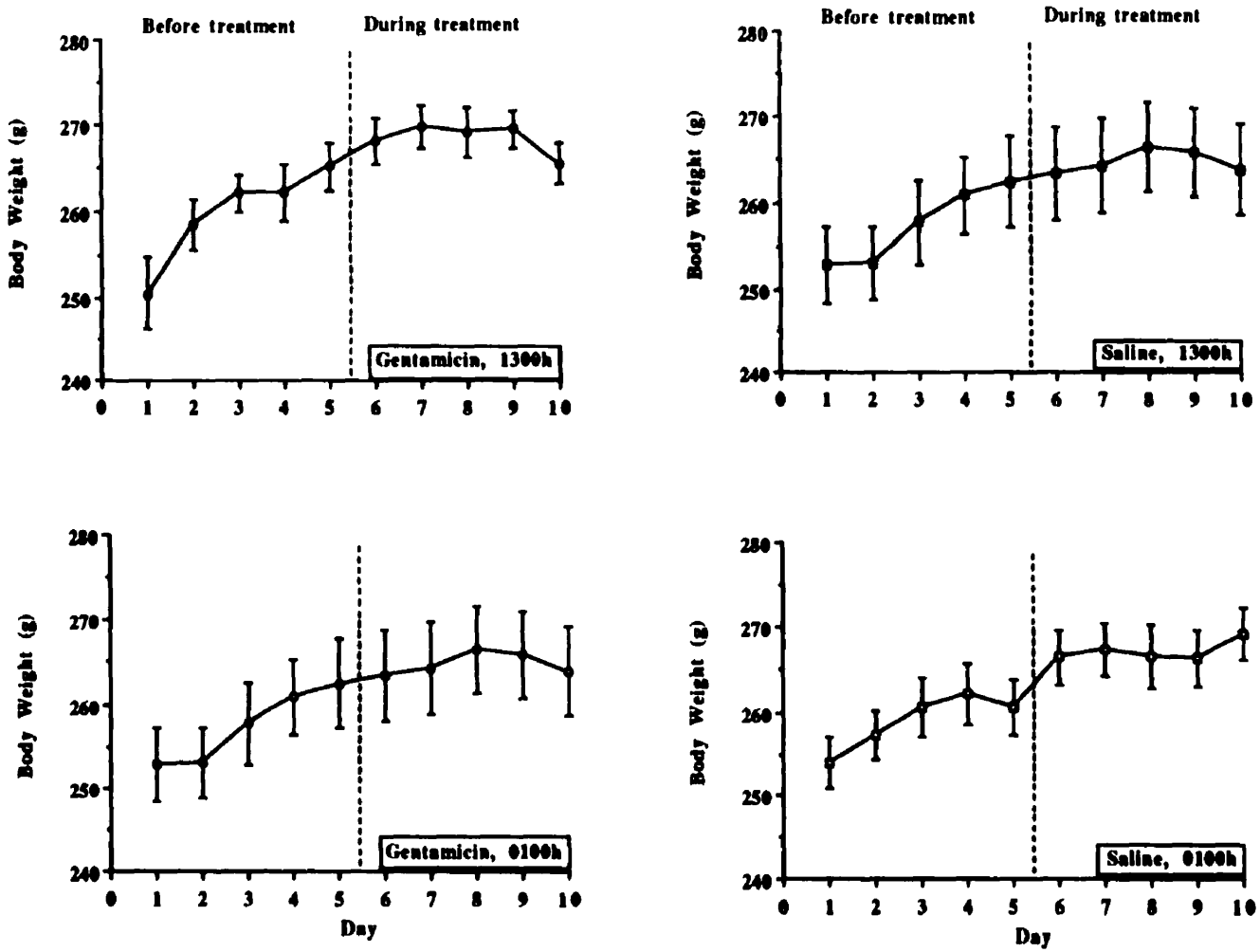


FIG. 2. Body weight of rats throughout the experimental period. Days 1 to 5 correspond to the pre-treatment period and days 6 to 10 correspond to the treatment period during which rats were treated at 1300 h or 0100 h with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See text for significance.

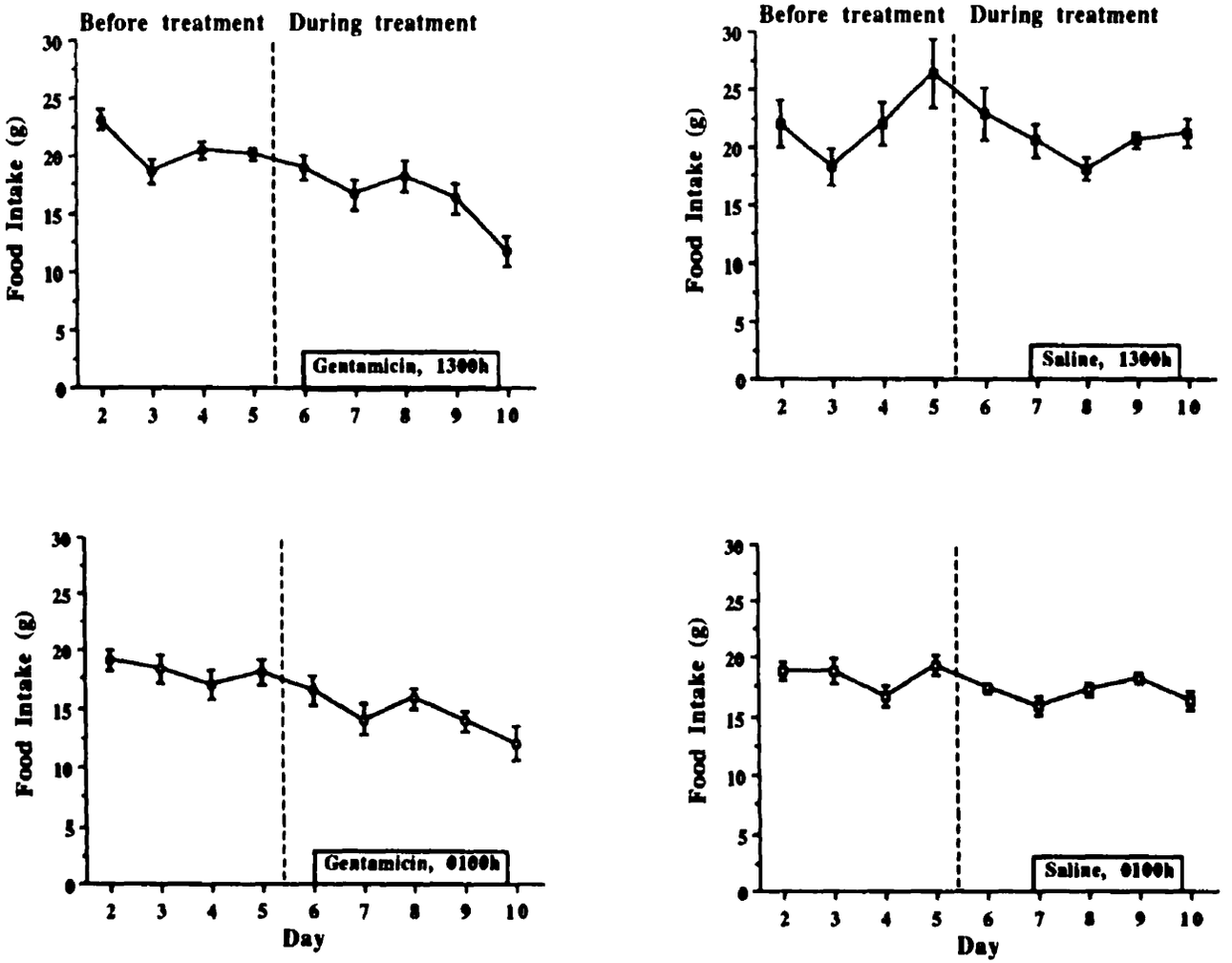


FIG. 3. Food intake over 24 h throughout the experimental period. Days 1 to 5 correspond to the pre-treatment period and days 6 to 10 correspond to the treatment period during which rats were treated at 1300 h or 0100 h with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See text for significance.

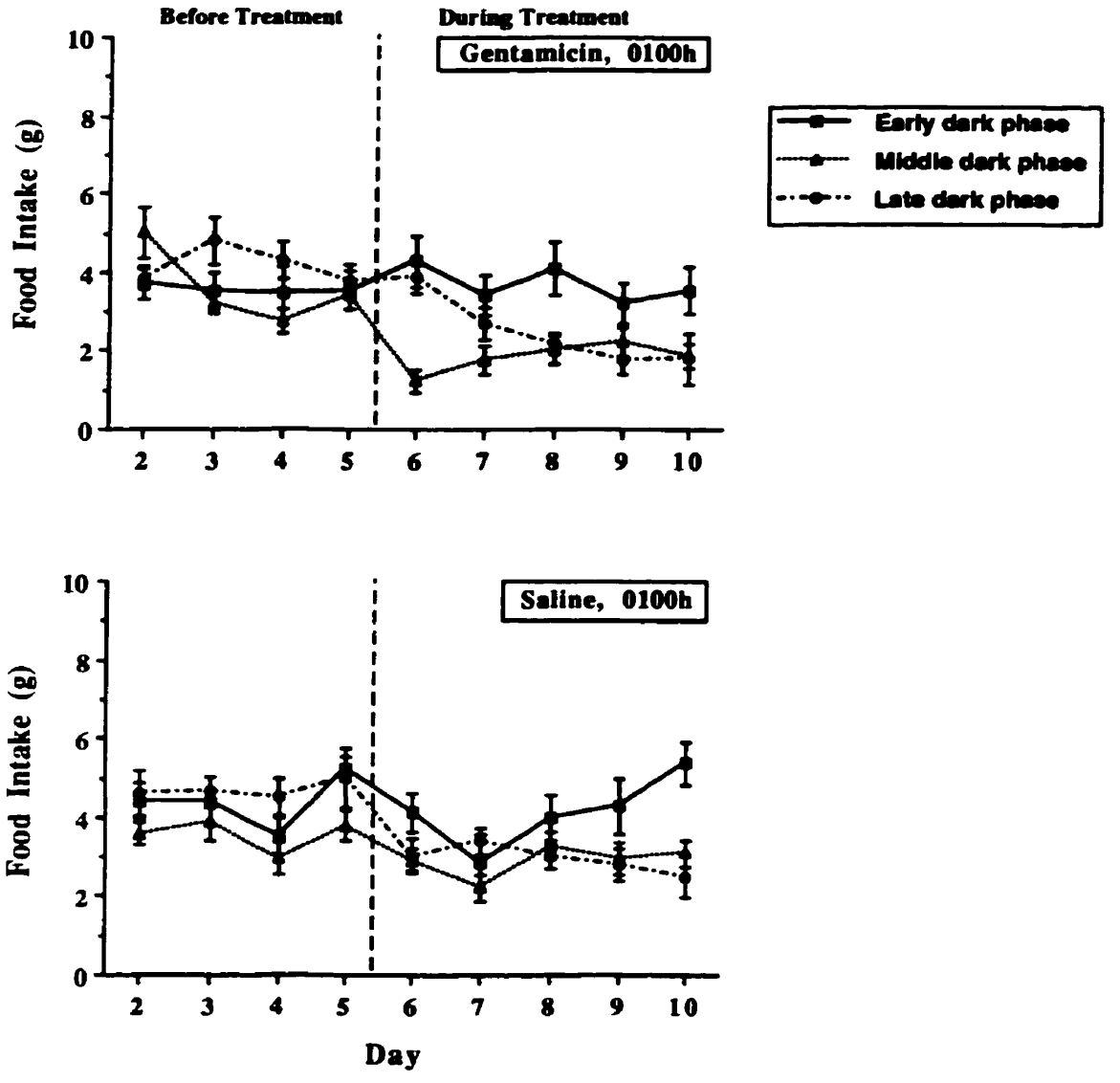


FIG. 4. Food intake during the early, middle, and late parts of the dark phase of the nycthemeral cycle throughout the experimental period. Days 1 to 5 correspond to the pre-treatment period and days 6 to 10 correspond to the treatment period during which rats were treated at 0100 h with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See text for significance.

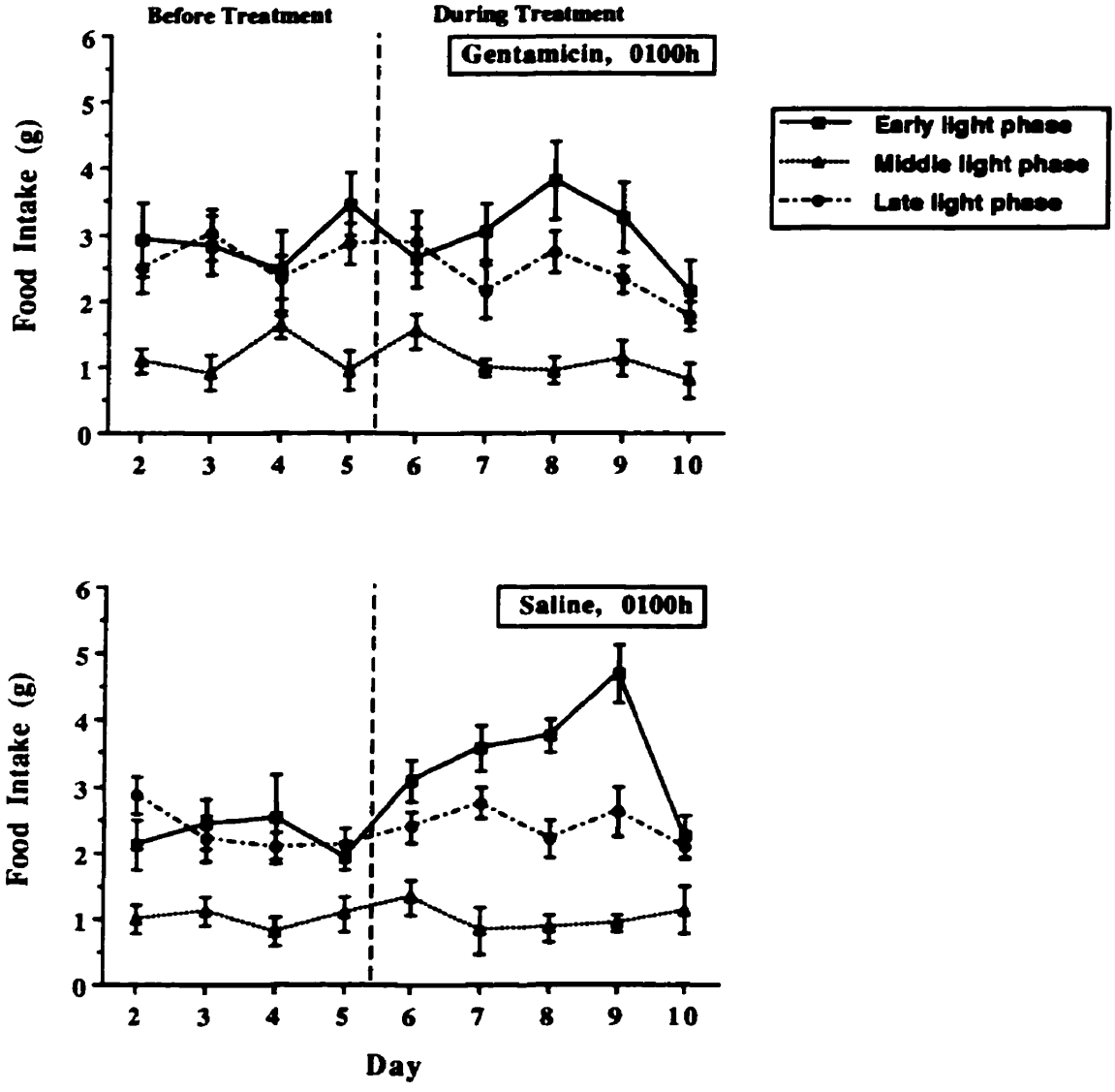


FIG. 5. Food intake during the early, middle, and late parts of the light phase of the nycthemeral cycle throughout the experimental period. Days 1 to 5 correspond to the pre-treatment period and days 6 to 10 correspond to the treatment period during which rats were treated at 0100 h with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See text for significance.

DISCUSSION

The results of the present study show for the first time that chronic gentamicin treatment, given at a dose of 80 mg/kg i.p. for 5 days to adult female Sprague-Dawley rats, resulted in an overall decrease in the 24 h food intake throughout the experimental period. In the group treated with gentamicin at 0100 h, the maximal food intake during the pre-treatment period was observed at late dark but during gentamicin administration a decrease in food intake occurred during that part of the dark phase. Maximal food intake was recorded at early dark rather than late dark. In addition, these results indicate that gentamicin treatment induced a nephrotoxicity as indicated by higher levels of cellular regeneration, higher 24 h urine output and increased urinary excretion of βGal, NAG, and γ-GT. Temporal variations in gentamicin nephrotoxicity was also demonstrated, with a higher level of serum creatinine in rats treated at 1300 h than at 0100 h, and by a lower rate of creatinine clearance in rats treated with gentamicin at 1300 h compared to animals that received saline at the same time.

The increase in serum creatinine and decrease in creatinine clearance in rats treated with gentamicin at 1300 h reported in the present study is in agreement with other animal studies reporting that nephrotoxicity was more important when AG were injected in the middle of the resting period than in the middle of the activity period. This was demonstrated using rats of different strains and of both sexes that were treated with different doses of various AG for variable number of days. For example, Yoshiyama et al. (1992) used adult male Wistar rats, adapted to a 12:12 light:dark cycle and treated with gentamicin (60 mg/kg/day, s.c.) for 8 days. Lin et al. (1994) and Yoshiyama et al. (1996a) utilized adult female Sprague-Dawley rats adapted to a 14:10 light:dark cycle and treated for 10 days with tobramycin (40 mg/kg/day, i.p.) or isepamicin (80 mg/kg/day, i.p.) respectively. Temporal variation in the nephrotoxicity of AG was also demonstrated in humans treated with gentamicin or tobramycin (standard dosage: 4 mg/kg, i.v.), with nephrotoxicity occurring significantly more often when the AG was given between 0000 h

and 0730 h (Prins et al., 1997). The results of the present investigation also suggest that the dose of 80 mg/kg/day of gentamicin induced a reduction of the glomerular filtration rate, thus causing damage at the glomerular level in addition to the tubular level. The nephrotoxicity was also assessed in the present work by determining the level of cellular regeneration and of urinary enzymes excretion. These parameters were not indicative of a more important renal toxicity when gentamicin was injected at 1300 h than at 0100 h. Yoshiyama et al. (1996a) also did not observe temporal variations in nephrotoxicity after 4 days of treatment with isepamicin at a dose of 80 mg/kg/day, i.p. administered at 1400 h and at 0200 h in male Sprague-Dawley rats. After 10 days of isepamicin treatment, however, rats injected at 1400 h had significantly higher levels of cellular regeneration than rats injected at 0200 h. Similarly, Lin et al. (1994) reported a temporal variation in the cellular regeneration after 10 days of treatment but not after 4 days, in female Sprague-Dawley rats injected daily with tobramycin at 40 mg/kg, i.p. Likewise, urinary enzymes are precocious indicators of renal toxicity, but in a recent study performed in our laboratory (unpublished data), a higher level of NAG excretion in the group treated at midlight compared to the one treated at middark, was observed after 7 days of treatment with gentamicin at 40 mg/kg/day i.p., in female Sprague-Dawley rats. Moreover, Yoshiyama et al. (1993) observed that the urinary NAG excretion started increasing significantly more in animals treated at midlight compared to the ones treated at middark only after 7 days of treatment with isepamicin for 15 consecutive days (300 mg/kg/day, i.p.). The short treatment period of 5 days with a low dose of 80 mg/kg used in the present study, could thus explain the absence of temporal variation for these two nephrotoxicity parameters. We did not observe a significant difference in the gentamicin intra-cortical accumulation between the groups treated at 1300 h and 0100 h. Similarly to our results, this was also reported in the study by Lin et al. (1994) despite the fact that other parameters such as cellular regeneration had shown a temporal variation of nephrotoxicity following a 10-day treatment with tobramycin (40 mg/kg, i.p.). This

suggest that the renal accumulation of gentamicin is not always a good indicator of renal toxicity. This could be explained by the fact that since the toxicity is higher when the antibiotic is injected at 1300 h, more cells are damaged and therefore sloughed away, whereas more cells remain intact when the antibiotic is injected at 0100 h, making this assay inaccurate.

The kidney has the ability to recover after being damaged, so that in AG-induced renal toxicity, acute tubular necrosis develops when cellular regeneration can no longer compensate for focal necrosis (Tulkens, 1986; Kaloyanides, 1991). The process of cellular regeneration has been demonstrated to display a diurnal fluctuation in different organs. In a study performed by Burns et al. (1972), the uptake of [^{3}H]-thymidine by the kidney of male mice fed ad libitum was maximal between 0100 h and 0500 h with a peak at 0100 h. Similarly, Dallman et al. (1974), showed that a maximal incorporation of [^{3}H]-thymidine into the nuclear DNA in liver of Sprague-Dawley rats in the dark phase, with a peak at 0400 h. These findings could partly explain why in the present work the nephrotoxicity was minimal when gentamicin was administered in the middle of the dark phase, period of maximal DNA synthesis.

In rodents fed ad libitum, the alternance of light and dark entrains a circadian rhythm of feeding and drinking behavior. Rats are nocturnal animals and therefore when maintained on a mixed complete diet and on 12:12 light:dark cycle, male rats eat about 70-85% of their total food intake (Le Magnen and Tallon, 1966; Johnson and Johnson, 1990; Larue-Achagiotis, 1992) and 85-92% of their total water intake (Fitzsimons and LeMagnen, 1969; Johnson and Johnson, 1990) during the dark phase. The present results indicate that our rats exhibited a normal temporal pattern of food and water intakes that varied across the nycthemeral cycle with the night-time intakes being higher than the day-time ones. However, the overall total daily food intake ranged from 59% to 67% and the total daily water intake ranged from 69% to 74% during the dark period in the four experimental groups, thus being slightly lower than the minimum 70% for food intake and

85% for water intake normally observed in the literature for nocturnal intakes. This difference could be partly explained by the fact that treating animals with gentamicin or saline during the period of maximal food and water intakes disturbs them and therefore decreases the percentage of ingestions taken during that period, and partly by the fact that we used female rats exposed to a dark phase of 10 h instead of the 12 h usually utilized in feeding studies conducted mainly on male rats.

The nocturnal feeding activity of rats has been shown to be bimodally distributed with maximums of food intake at the beginning and end of the phase and a minimum in the middle (Siegel, 1961). Consistent with this finding, during the pre-treatment period, animals displayed peaks of food intake at early and late dark and a trough at middark. However, during gentamicin treatment at 0100 h, more specifically on days 8 to 10, this bimodal distribution was disrupted. In fact, only one peak of food intake at early dark was observed. The maximum intake observed at late dark during the pre-treatment period disappeared due to a marked decrease in food intake during that part of the dark phase. The effect of the treatment was thus present not only when the drug was administered, that is at middle dark, but also during the following part, that is late dark.

Animals treated with saline at 0100 h increased their food intake in the early part of the light phase, resulting in an overall maintenance of the total food intake throughout the experimental period that was contrary to the animals treated with gentamicin. This compensatory behavior that took place during the light phase to make up for the decrease in food intake that occurred during the dark phase was not observed in animals treated with gentamicin. The animals treated at 0100 h were subjected to the acute physical stress of injection and of manipulation by the experimenter during a period of intense activity and feeding. Such stress caused a decrease in food intake in the hours following the exposure but did not have lasting effect in saline-treated rats at 0100 h, so that animals managed to make up for the decrease in food intake. The animals treated with gentamicin, however,

were subjected to an additional stress, that is the chronic metabolic stress of gentamicin action and of gentamicin-induced renal toxicity.

In all experimental groups, the overall 24 h water intake remained stable throughout the experimental period. However, considering the overall decrease in food intake during the gentamicin treatment period, a decrease in water intake would have been expected to accompany this decrease since the majority of water consumption is closely associated with food intake (Fitzsimons and LeMagnen, 1969; Johnson and Johnson, 1990). Gentamicin treatment resulted in a significant increase in urine output, consequently animals may have responded to this increased fluid loss by maintaining their water intake despite the decrease in food intake.

The findings of the present study support previous studies done in our laboratory (Beauchamp et al., 1996; Beauchamp et al., 1997) indicating the importance food plays in modulating the temporal variations of AG-induced nephrotoxicity. The process by which food is protective is not well understood but it could increase drug clearance and stimulate renal tubular cell regeneration among other things. Considering the fact that gentamicin inhibits food intake, as shown in this investigation, we suggest that this decrease of food intake may exacerbate the nephrotoxic effect of this drug.

Chapter 4

GENERAL CONCLUSION

To the knowledge of the investigators, this study was the first to report on the effect of gentamicin treatment on food and water intakes of rats. It demonstrated that treating the animals with gentamicin with a dose of 80 mg/kg administered i.p. for five consecutive days at 1300 h or 0100 h, resulted in an overall decrease in the 24 h food intake, altered its nocturnal variations, but had no effect on the overall water intake. The present results also confirmed the literature indicating a temporal variation in gentamicin-induced nephrotoxicity, with a maximum nephrotoxicity observed when the drug was injected in the middle of the animal's resting period and a minimum nephrotoxicity when injected in the middle of the animal's activity period.

A limitation of the present study is the length of the treatment period. Due to time constraint, a five day treatment period was chosen, but it may not have been long enough to observe the full effect of gentamicin treatment on food intakes and on the toxicity parameters of cellular regeneration and urinary enzymes excretion. Although this was a sufficient period to observe a significant reduction in the overall 24 h food intake, it would have been interesting to observe the outcome of a longer treatment, since the inhibition of food intake became more pronounced on the last two days of treatment.

We suggest that a decrease in food intake by gentamicin treatment could exacerbate its nephrotoxicity. In order to test this hypothesis, an experiment in which animals would be force-fed, in order to maintain the food intake constant throughout the treatment period, should be conducted.

Results from this study lead to new questions for future investigations such as can macronutrient-rich diets be protective against gentamicin-induced nephrotoxicity? If yes, what are the optimal sources and levels? It is known that food affects protein synthesis indirectly through changes in hormonal balance (Groff et al., 1995). In general, protein

synthesis is promoted by insulin (Groff et al, 1995). In man, insulin secretion is influenced by food intake and by an endogenous circadian rhythm (Reinberg et al., 1991). The protein component of food intake is partly responsible for insulin secretion, and therefore of particular importance for protein synthesis. Administering casein or its equivalent amino acids to female Fisher rats that had been fed a protein-free diet for three days increased liver DNA synthesis as demonstrated by the uptake of [^{3}H]-thymidine (Short et al., 1973). The protein fraction of food also has an effect on the glomerular filtration rate. In humans, protein uptake increases renal blood flow and GFR following a protein meal, with the rise being maximal after two and a half hours. A previous study done in our laboratory (Lin et al., 1994) showed a significantly higher clearance rate of gentamicin in the group of rats injected at 0200 h compared with the one injected at 1400 h. This can be explained by the circadian rhythm of GFR as demonstrated by a peak in the renal inulin clearance between 0000 h and 0400 h in rats (Pons et al., 1984). The increase in GFR caused by food may have helped in increasing gentamicin clearance.

It is difficult to extrapolate results from animal studies to humans situations, but in the light of the results of the present investigation we recommend that patients under an AG treatment regimen be treated in the middle of their activity period and that their food intake be maintained, possibly with the help of a nutritional supplement. Taking into consideration the results of the present study and answering the proposed questions would hopefully lead to a better understanding of the relationship between diet and AG-induced nephrotoxicity, and eventually result in the development of new therapeutic approaches that will benefit the patients.

TABLE A1. Factorial ANOVA of nephrotoxicity parameters in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h.

STATISTICAL ANALYSIS

Nephrotoxicity Parameters		Main Effects: Treatment[1] F value	Treatment[1] probability	Injection Time[2] F value	Injection Time[2] probability	Treatment*Injection Time[3] F value	Treatment*Injection Time[3] probability
Cellular Regeneration	ANOVA[4]	(1,29)=21.746	0.0001	(1,29)=0.141	0.7101	(1,29)=0.623	0.4362
Serum Creatinine	ANOVA	(1,32)=6.476	0.0160	(1,32)=5.370	0.0270	(1,32)=1.465	0.235
Blood Urea Nitrogen	ANOVA	(1,32)=0.563	0.4584	(1,32)=2.560	0.1195	(1,32)=0.109	0.7434
Creatinine Clearance	ANOVA	(1,32)=10.586	0.0027	(1,32)=1.554	0.2215	(1,32)=4.151	0.0400
Diuresis	ANOVA	(1,32)=5.033	0.0319	(1,32)=0.945	0.3382	(1,32)=0.001	0.9751
βGal	ANOVA	(1,31)=12.486	0.0013	(1,31)=2.652	0.1136	(1,31)=0.061	0.8062
NAG	ANOVA	(1,31)=10.810	0.0025	(1,31)=1.152	0.2914	(1,31)=0.025	0.8744
γ-GT	ANOVA	(1,30)=11.667	0.0018	(1,30)=0.079	0.7803	(1,30)=0.664	0.4215
Gentamicin Cortical Levels	ANOVA	-	-	(1,16)=3.728	0.0714	-	-

[1] Treatment main effect (gentamicin and saline)
[2] Injection time main effect (1300 h and 0100 h)
[3] Treatment-by-injection time interaction
[4] ANOVA is restricted to the between-subject effects (i.e., treatment, injection time, and treatment-by-injection time); a rat is a "subject".

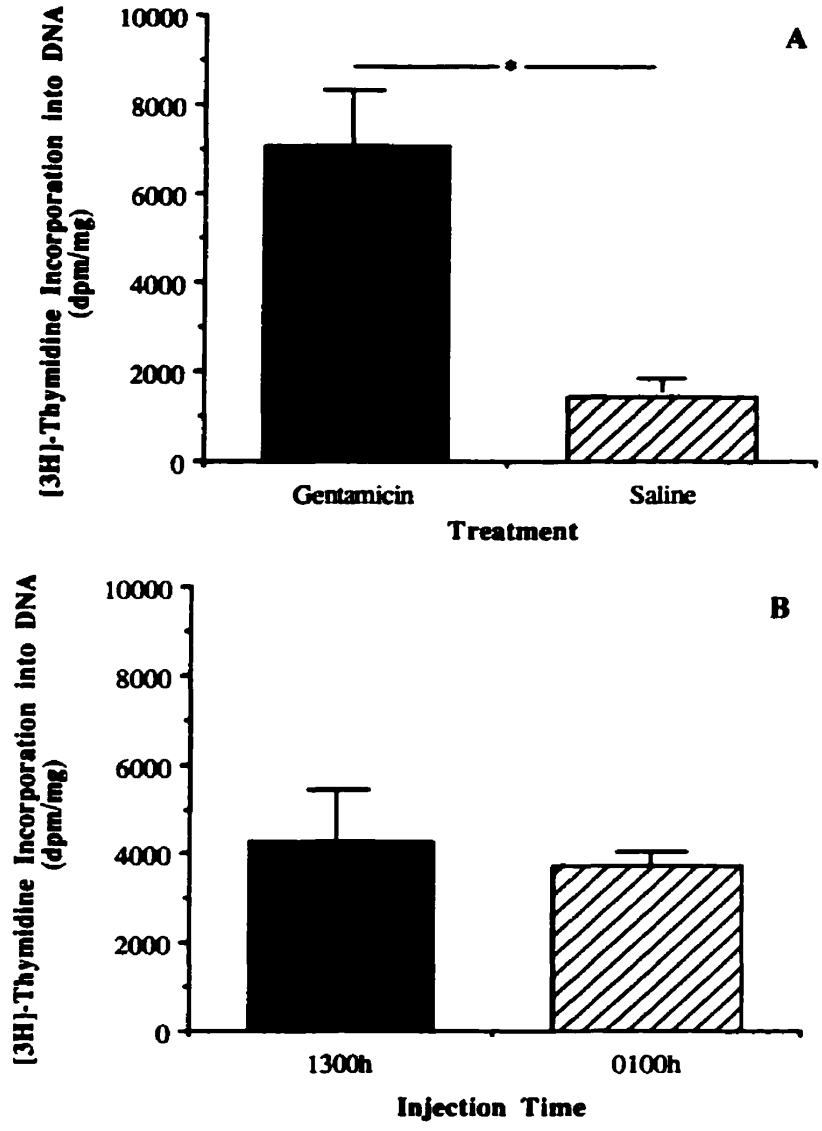


FIG. A1a. The effect of treatment (A) and injection time (B) on [^{3}H]-thymidine incorporation in the renal cortex DNA of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM. * Significant difference at $p<0.05$ as determined by two-way ANOVA.

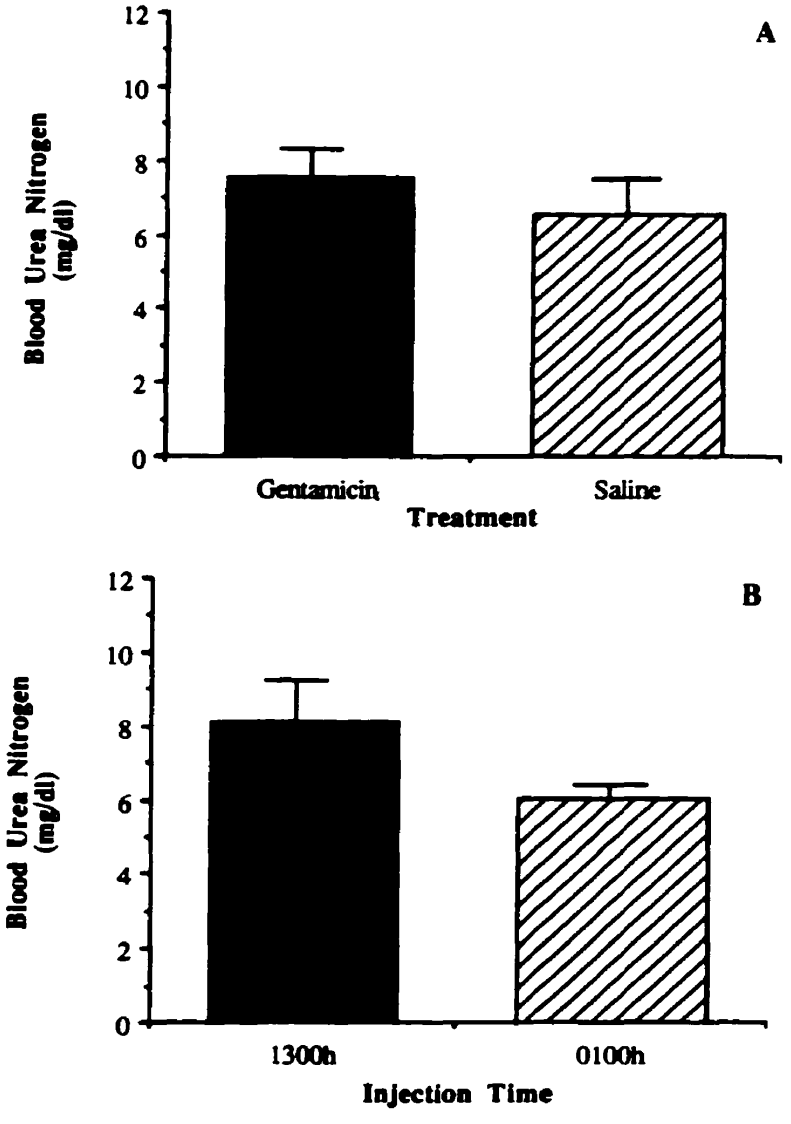


FIG. A1b. The effect of treatment (A) and injection time (B) on blood urea nitrogen levels of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM.

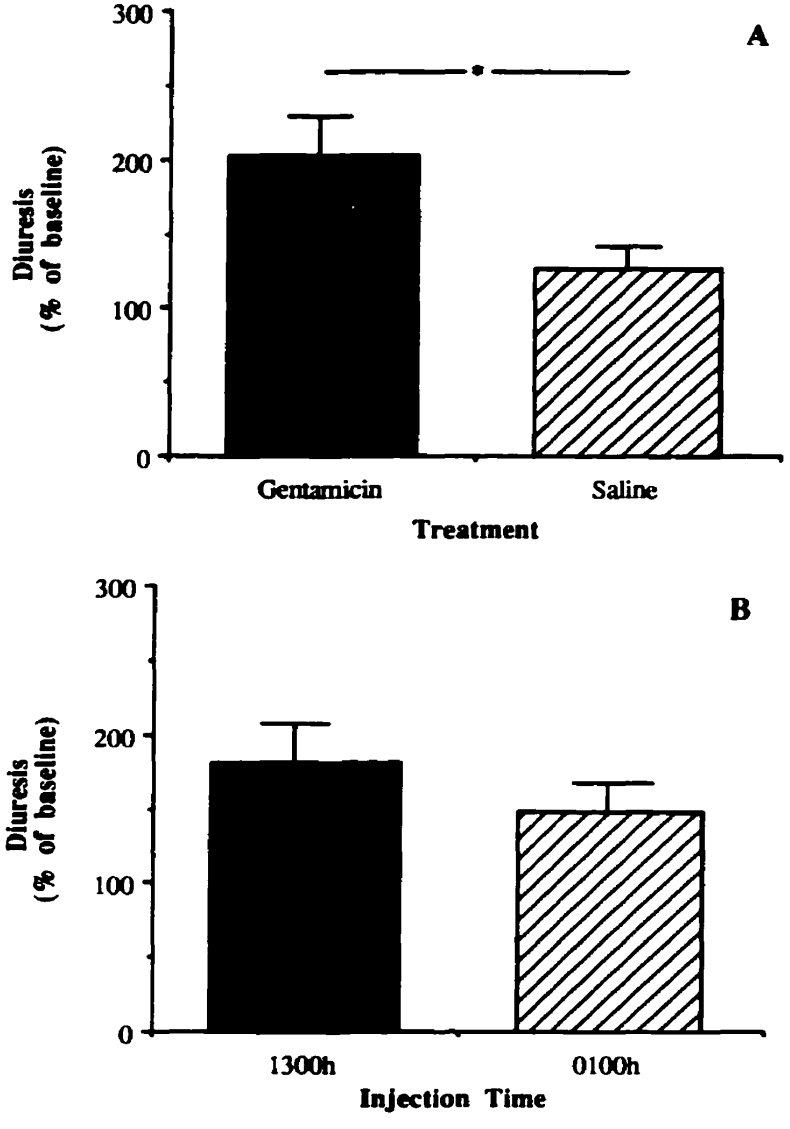


FIG. A1c. The effect of treatment (A) and injection time (B) on the 24 h diuresis of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM. * Significant difference at $p<0.05$ as determined by two-way ANOVA.

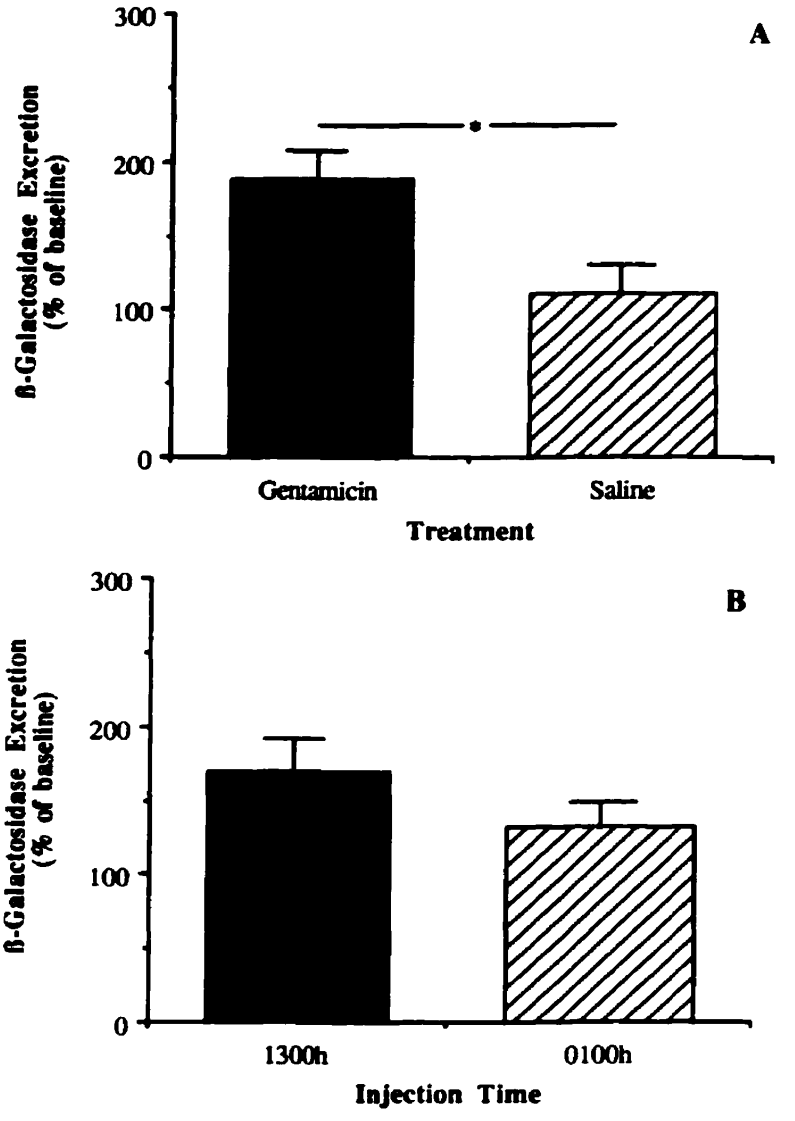


FIG. A1d. The effect of treatment (A) and injection time (B) on the urinary ß-galactosidase excretion of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM. * Significant difference at $p<0.05$ as determined by two-way ANOVA.

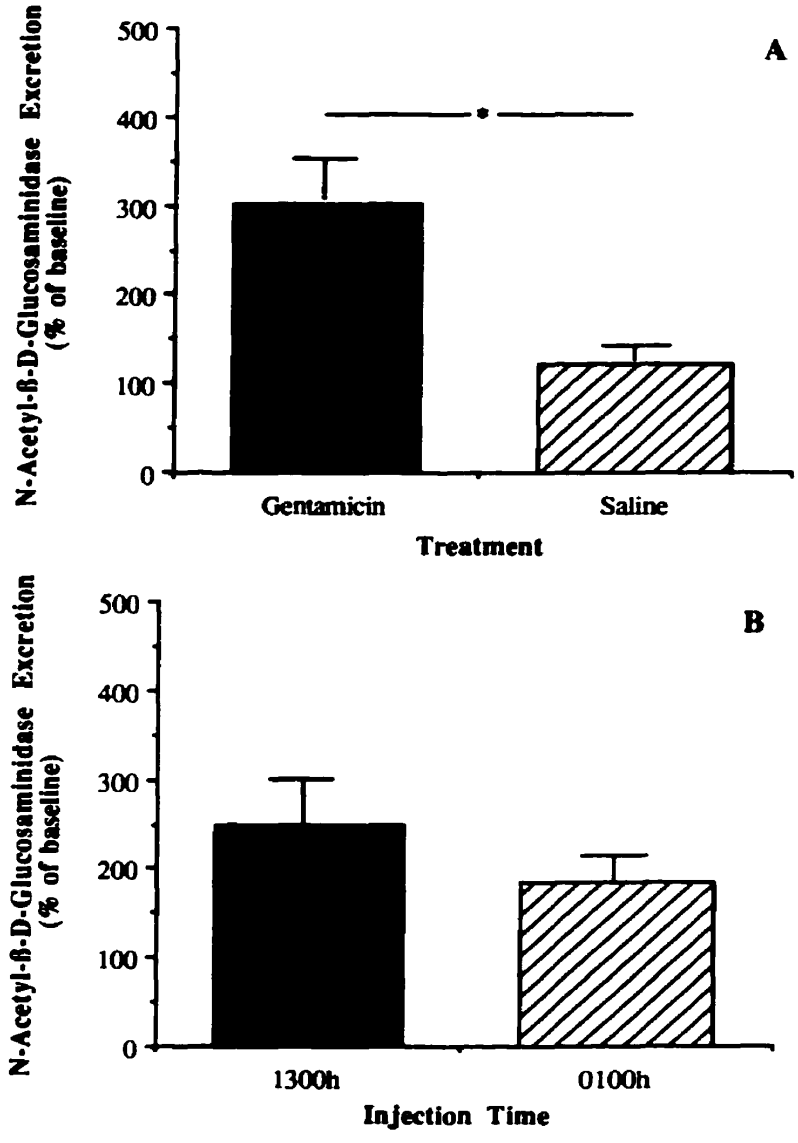


FIG. A1e. The effect of treatment (A) and injection time (B) on the urinary N-acetyl-ß-D-glucosaminidase excretion of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM. * Significant difference at $p<0.05$ as determined by two-way ANOVA.

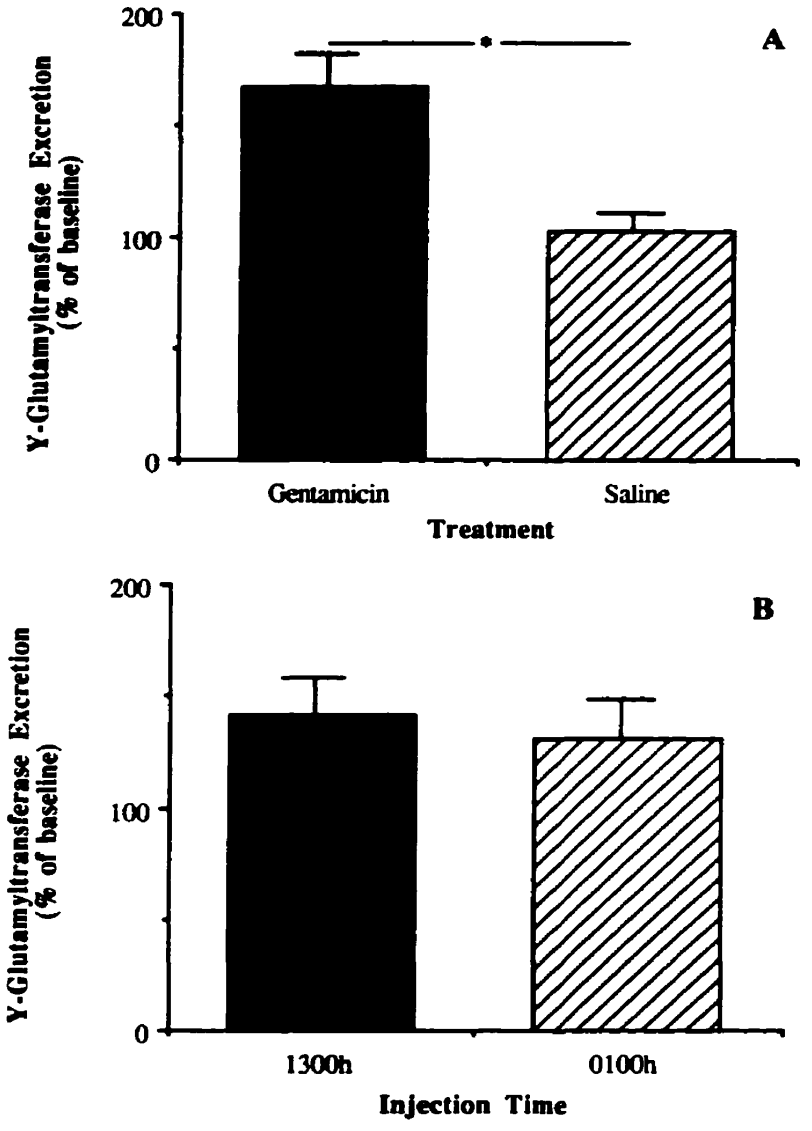


FIG. A1f. The effect of treatment (A) and injection time (B) on the urinary γ-glutamyltransferase excretion of rats treated for five days with gentamicin (80 mg/kg, i.p.) or saline at 1300 h or 0100 h. Data expressed as mean ± SEM. * Significant difference at $p<0.05$ as determined by two-way ANOVA.

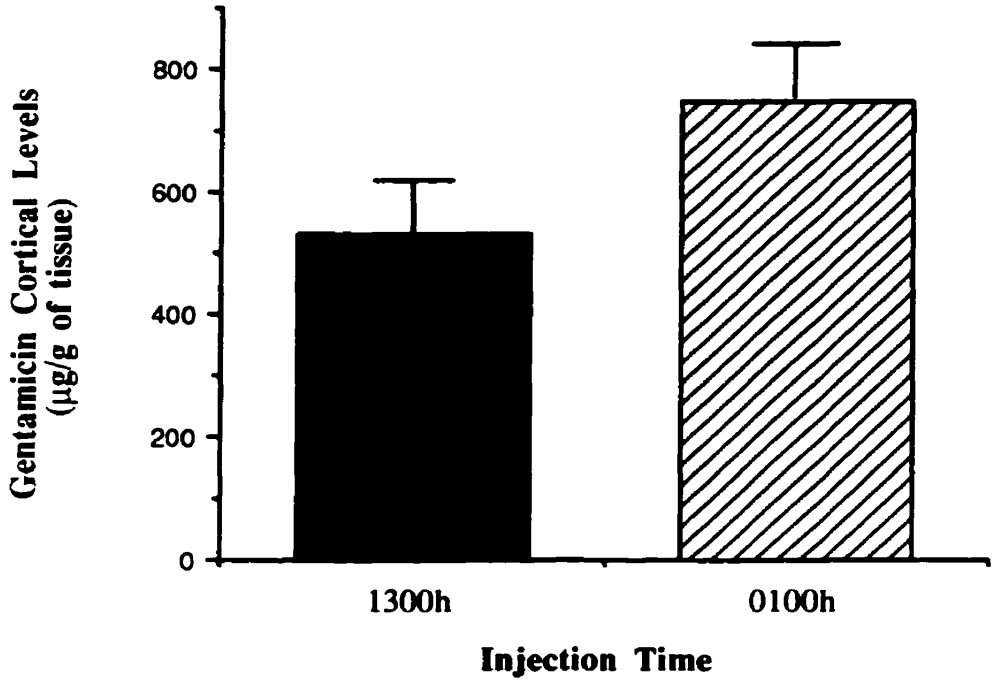


FIG. A1g. The effect of injection time on gentamicin cortical levels following five daily injections of gentamicin (80 mg/kg, i.p.) given at 1300 h or 0100 h. Data expressed as mean ± SEM.

TABLE A2. Repeated measures ANOVA of body weight gain (g/day) of rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h.

STATISTICAL ANALYSIS

Groups			Main Effect Day[1] F value	probability	Epsilon
Gentamicin, 1300h	Modified ANOVA[2]	G-G	(8,64)=2.696	0.0619	0.410
		H-F		0.0258	0.730
Saline, 1300h	Modified ANOVA	G-G	(8,64)=0.640	0.5913	0.361
		H-F		0.6611	0.587
Gentamicin, 0100h	Modified ANOVA	G-G	(8,64)=1.416	0.2606	0.398
		H-F		0.2335	0.690
Saline, 0100h	Modified ANOVA	G-G	(8,64)=2.685	0.0860	0.298
		H-F		0.0587	0.434

[1] Day main effect (days 2 to 10)

[2] The within-subject effect (i.e., day main effect) is tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

TABLE A3. Repeated measures ANOVA of food intake (g) over 24 h in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effect of day.

STATISTICAL ANALYSIS

Groups			Main Effect Day[1] F value	probability	Epsilon
Gentamicin, 1300h	Modified ANOVA[2]	G-G	(8,56)=8.692	0.0020	0.290
		H-F		0.0002	0.443
Saline, 1300h	Modified ANOVA	G-G	(8,40)=3.057	0.0912	0.253
		H-F		0.0517	0.425
Gentamicin, 0100h	Modified ANOVA	G-G	(8,64)=7.77	0.0005	0.419
		H-F		0.0001	0.756
Saline, 0100h	Modified ANOVA	G-G	(8,56)=3.362	0.0712	0.356
		H-F		0.0539	0.625

[1] Day main effect (days 2 to 10)

[2] The within-subject effect (i.e., day main effect) is tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

TABLE A4. Repeated measures ANOVA of food intake (g) over 24 h in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,15)=10.087	0.0063					
	Modified ANOVA[5]	G-G			(4,60)=6.139	0.0010	(4,60)=7.320	0.0003	0.811
		H-F				0.0003		0.0001	1.130
0100h	ANOVA		(1,16)=6.859	0.0186					
	Modified ANOVA	G-G			(4,64)=6.971	0.0009	(4,64)=3.665	0.0223	0.682
		H-F				0.0001		0.0128	0.888

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

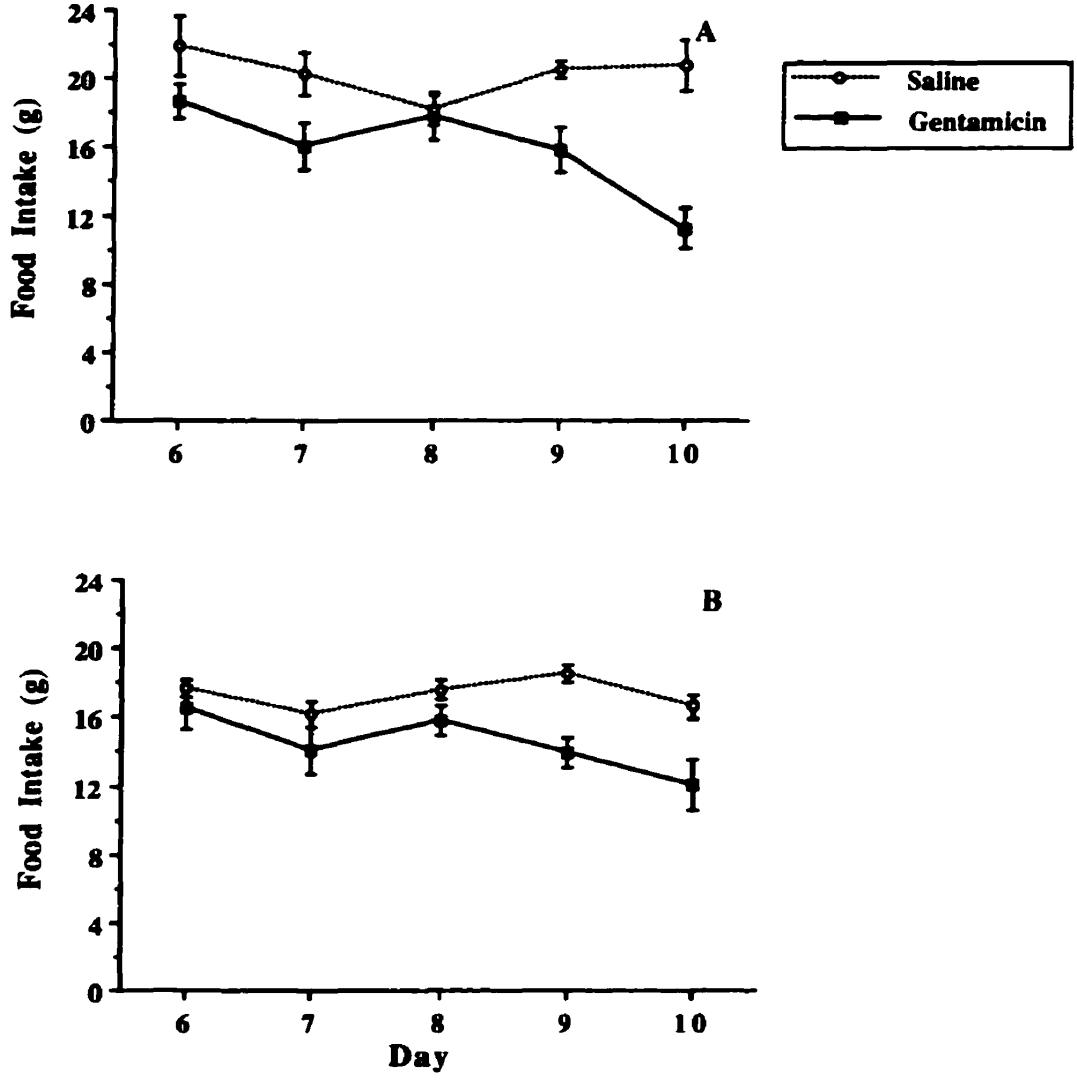


FIGURE A4a. The effect of day-by-treatment on food intake over 24 h in rats treated at 1300 h (A) or 0100 h (B) with gentamicin (80 mg/kg, i.p.) or saline (B). Data expressed as mean ± SEM. See table Table A4 for significance.

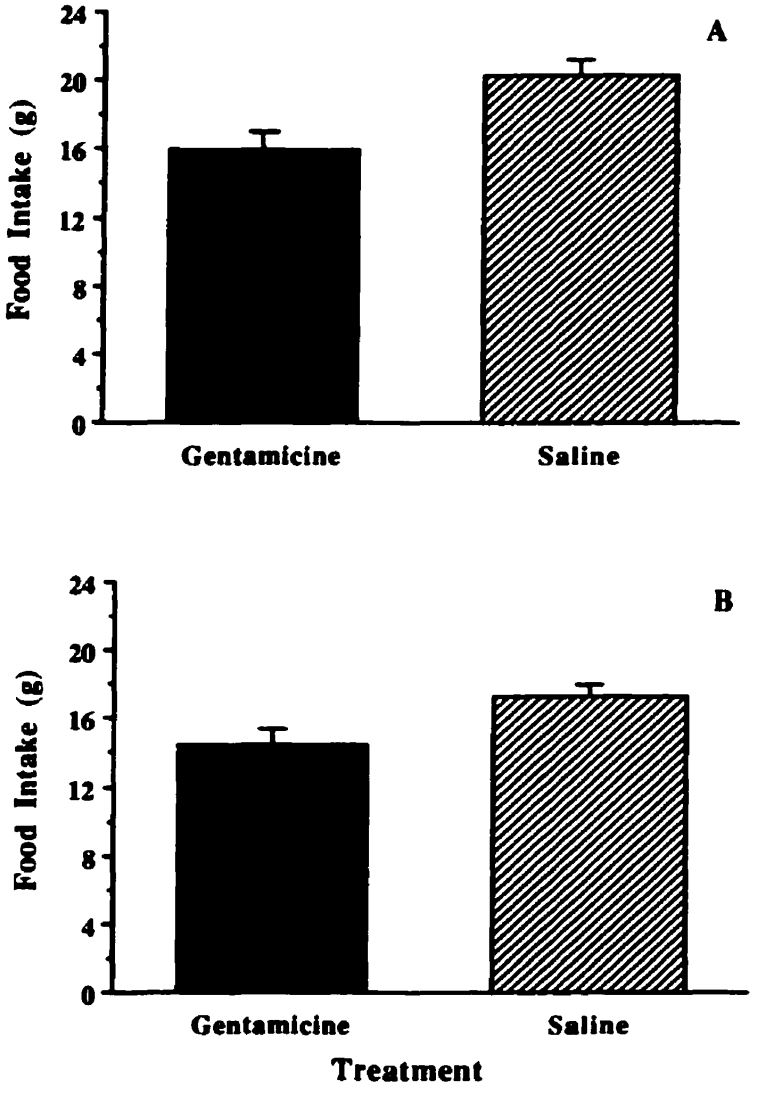


FIGURE A4b. The effect of treatment on the 24 h food intake in rats treated at 1300 h (A) or 0100h (B) with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. In graphics 'A' and 'B', since there was a significant interaction between treatment and day, the difference between treatments is not shown.

TABLE A5. Repeated measures ANOVA of food intake (g) during the 14 h light and 10 h dark phases of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(1,12)=40.133	0.0001					
	Modified ANOVA[5]	G-G			(8,96)=5.568	0.0041	(8,96)=1.206	0.3211	0.345
		H-F				0.0010		0.3206	0.495
Saline, 1300h	ANOVA		(1,8)=14.308	0.0054					
	Modified ANOVA	G-G			(8,64)=1.975	0.1540	(8,64)=3.462	0.0394	0.329
		H-F				0.1118		0.0139	0.566
Gentamicin, 0100h	ANOVA		(1,10)=7.687	0.0197					
	Modified ANOVA	G-G			(8,80)=2.164	0.1045	(8,80)=1.587	0.2071	0.419
		H-F				0.0622		0.1700	0.719
Saline, 0100h	ANOVA		(1,14)=52.379	0.0001					
	Modified ANOVA	G-G			(8,112)=2.155	0.1101	(8,112)=10.520	0.0001	0.362
		H-F				0.0860		0.0001	0.500

[1] Phase main effect (14 h light and 10 h dark phases)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

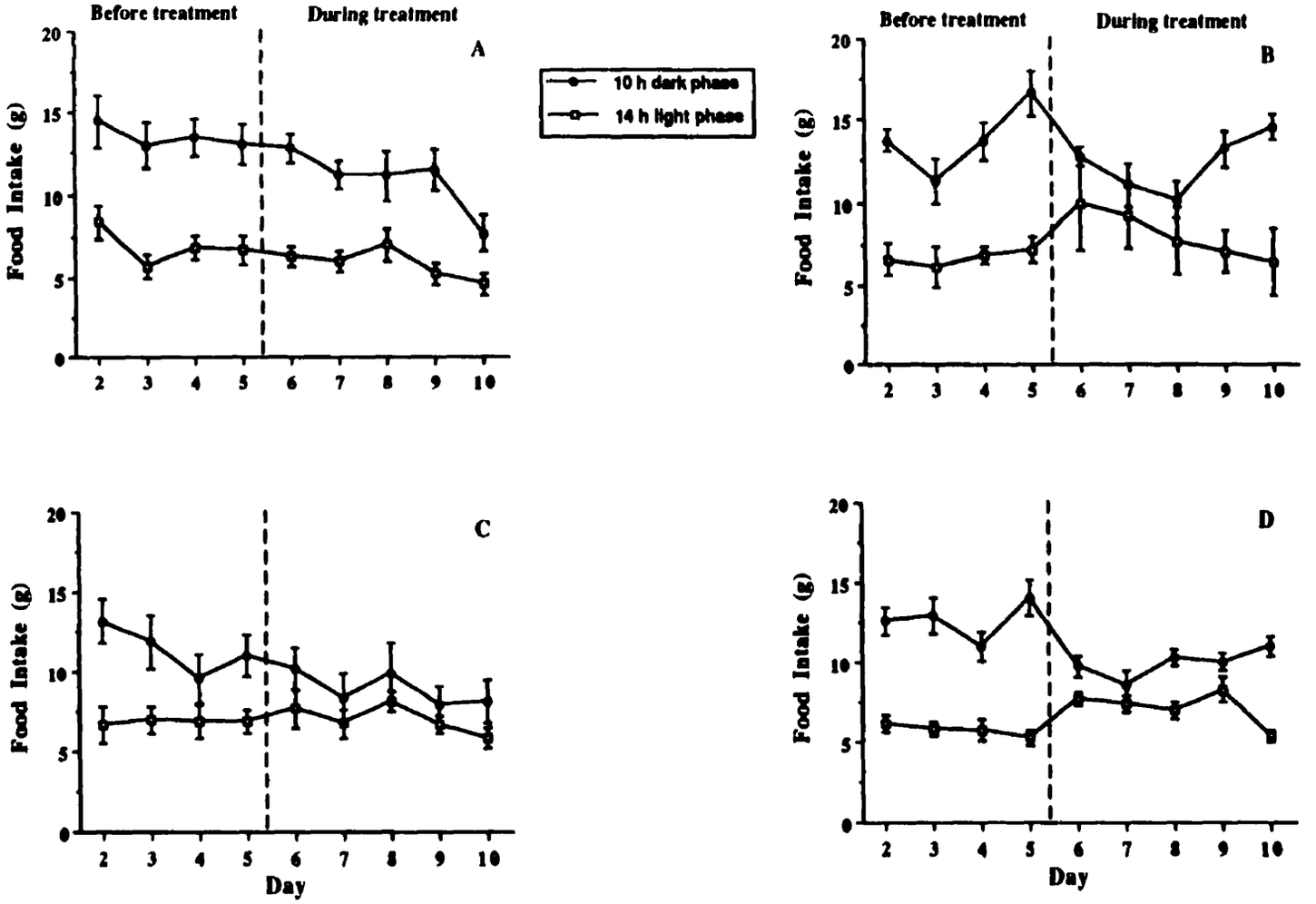


FIGURE A5a. The effect of day-by-phase on food intake during the 14 h light and 10 h dark phases of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See table Table 5 for significance.

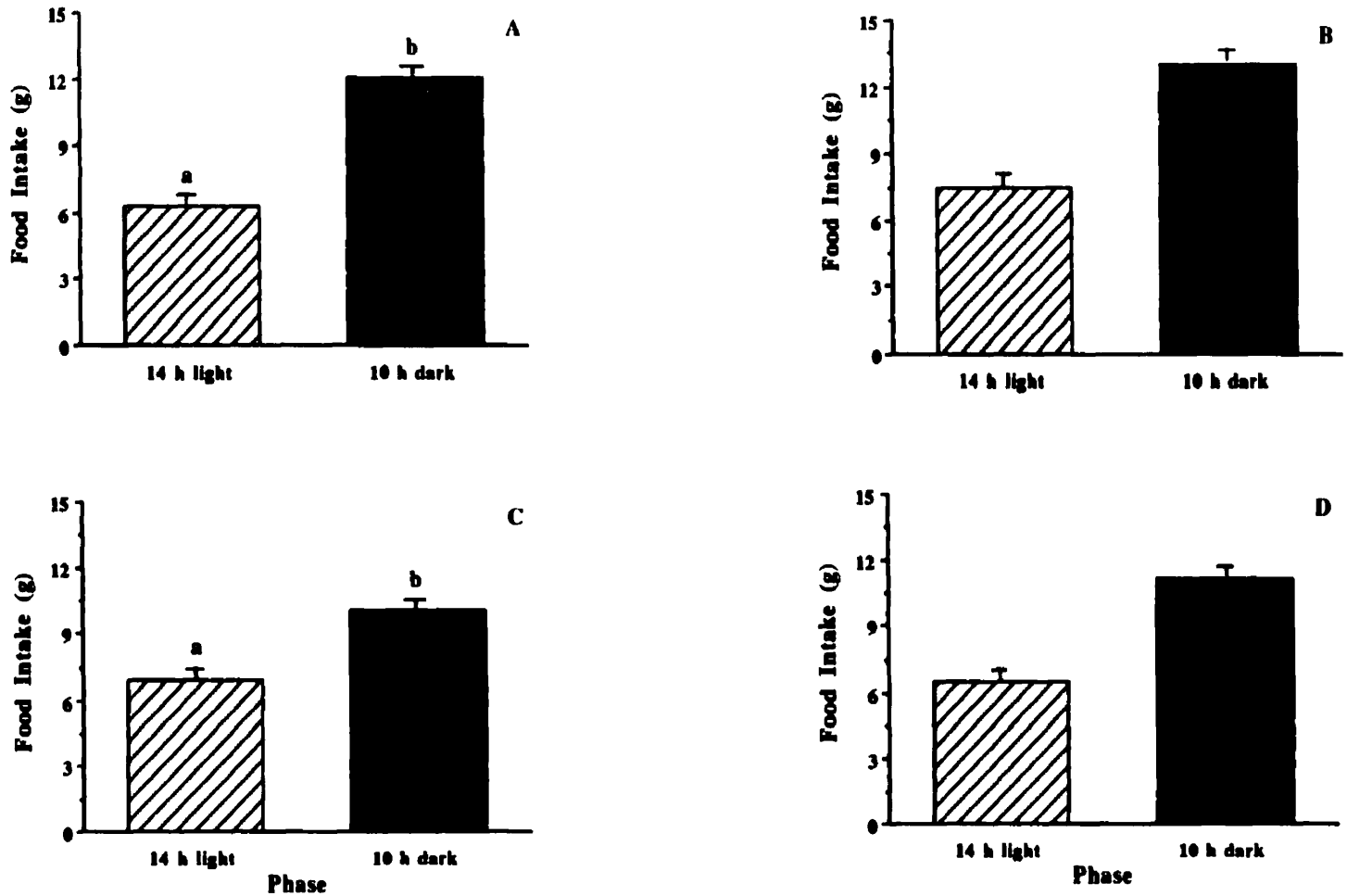


FIGURE A5b. The effect of the 14 h light and 10 h dark phases of the nycthemeral cycle on food in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. Columns within a group not sharing the same lower case letter are significantly different from one another at $p<0.05$. In graphics 'B' and 'D', since there was a significant interaction between phase and day, the difference between phases is not shown.

TABLE A6. Repeated measures ANOVA of food intake (g) during the 10 h dark phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,13)=2.634	0.1286					
	Modified ANOVA[5]	G-G			(4,52)=3.104	0.0490	(4,52)=8.73	0.0005	0.616
		H-F				0.0321		0.0001	0.829
0100h	ANOVA		(1,13)=1.358	0.2647					
	Modified ANOVA	G-G			(4,52)=1.619	0.2178	(4,52)=1.162	0.2097	0.494
		H-F				0.2091		0.2001	0.627

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

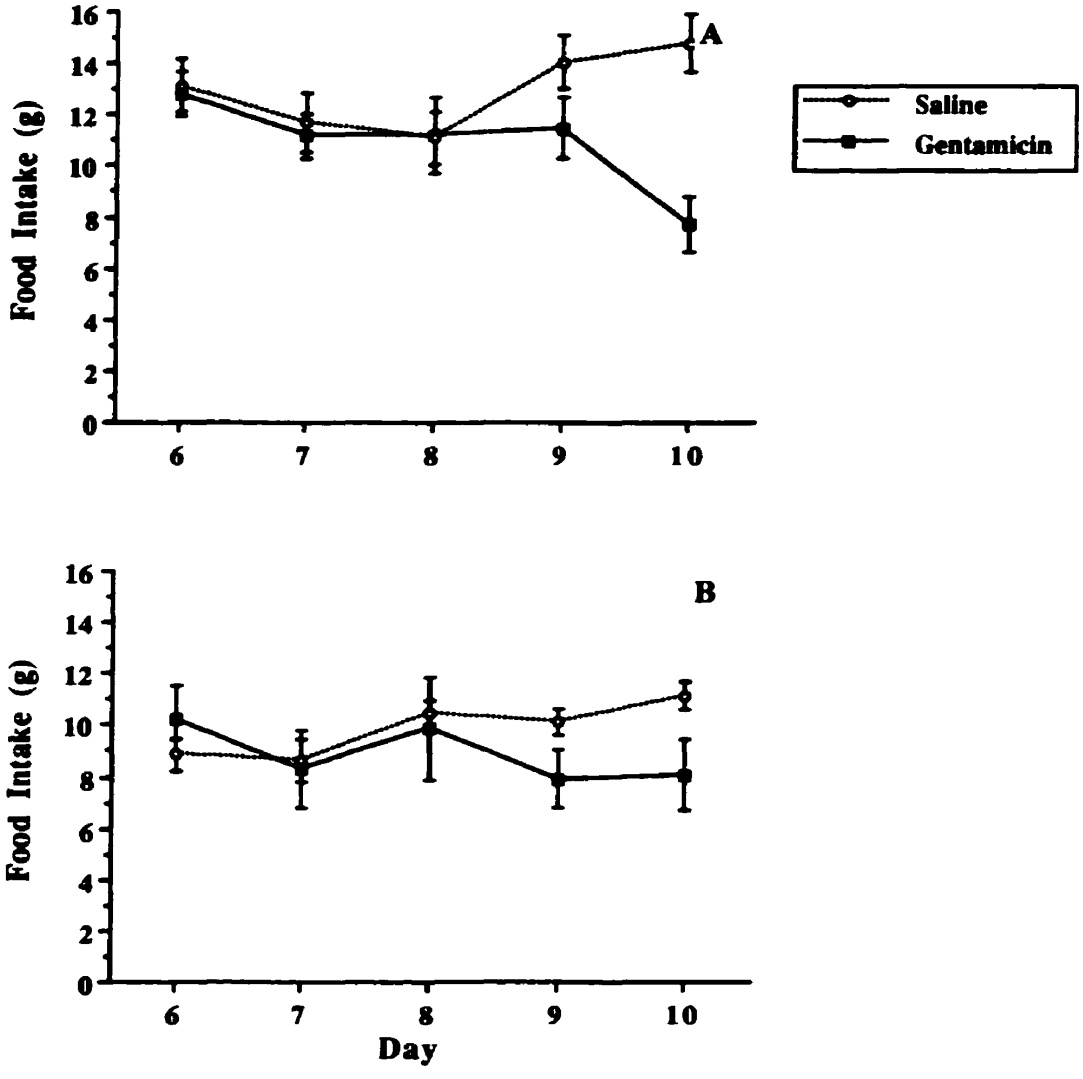


FIGURE A6a. The effect of day-by-treatment on food intake during the 10 h dark phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 h (B) with gentamicin (80 mg/kg, i.p) or with saline. Data expressed as mean ± SEM. See table Table A6 for significance.

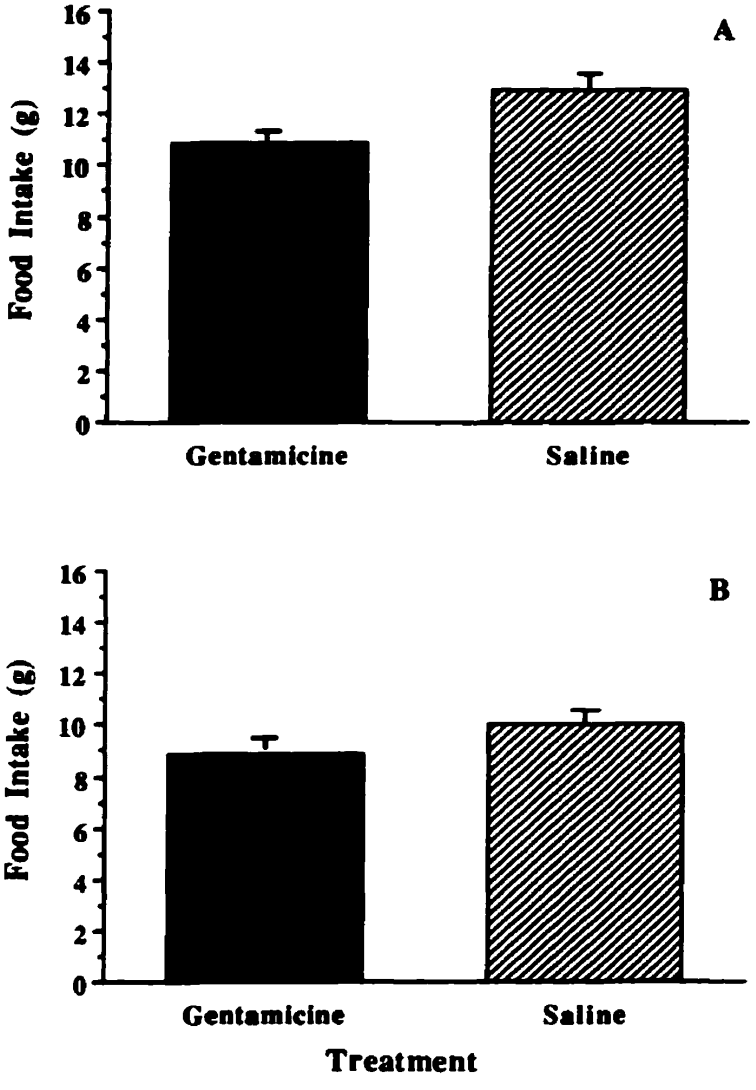


FIGURE A6b. The effect of treatment on food intake during the 10 h dark phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 (B) with gentamicin (80 mg/kg, i.p.) or with saline. Data expressed as mean ± SEM.

TABLE A7. Repeated measures ANOVA of food intake (g) during the 14 h light phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,13)=0.003	0.9576					
	Modified ANOVA[5]	G-G			(4,52)=3.755	0.1680	(4,52)=0.595	0.6287	0.783
		H-F				0.0093		0.6677	1.139
0100h	ANOVA		(1,14)=0.067	0.7966					
	Modified ANOVA	G-G			(4,56)=0.590	0.6580	(4,56)=0.487	0.7299	0.920
		H-F				0.6716		0.7450	1.377

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

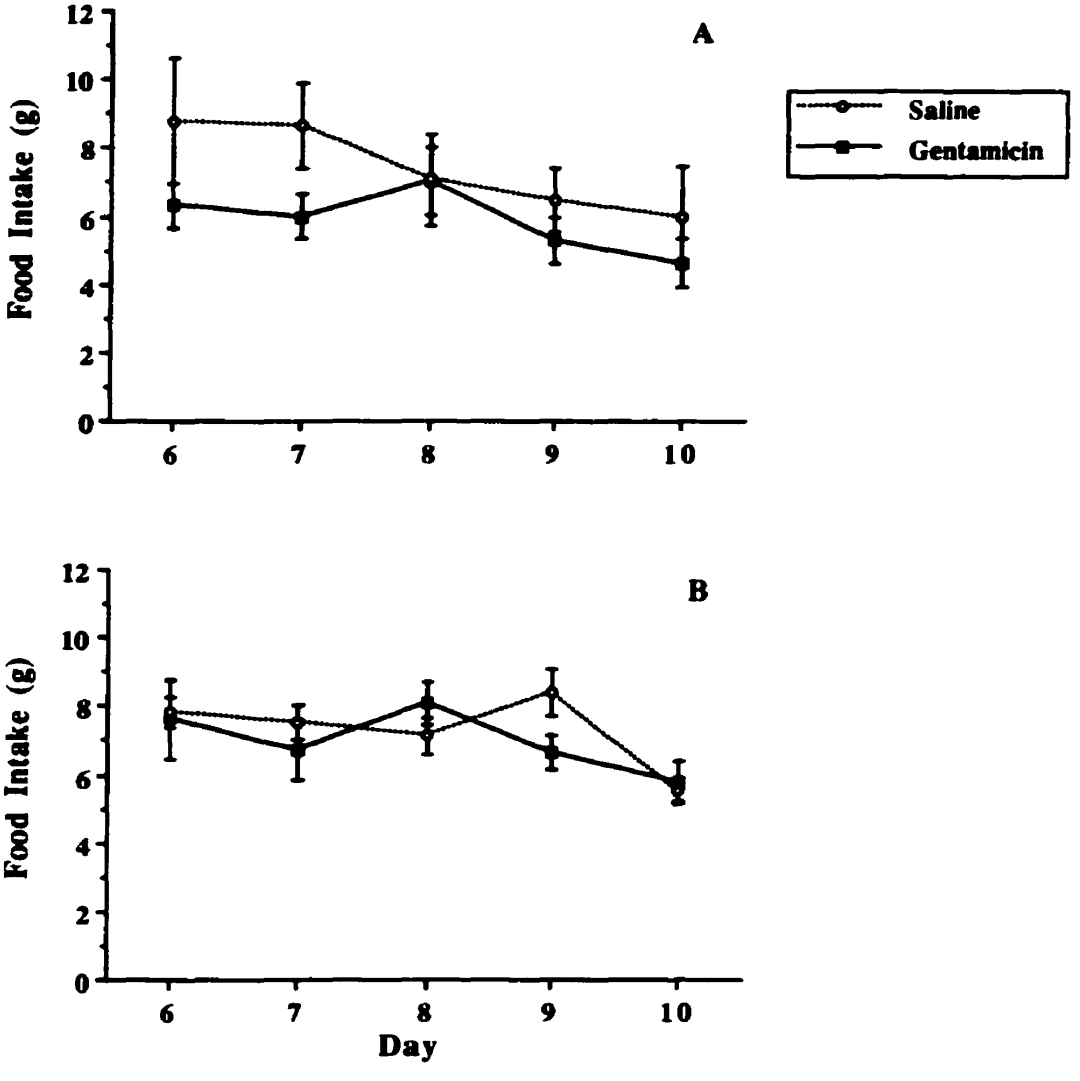


FIGURE A7a. The effect of day-by-treatment on food intake during the 14 h light phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 h (B) with gentamicin (80 mg/kg, i.p.) or with saline. Data expressed as mean ± SEM. See table Table A7 for significance.

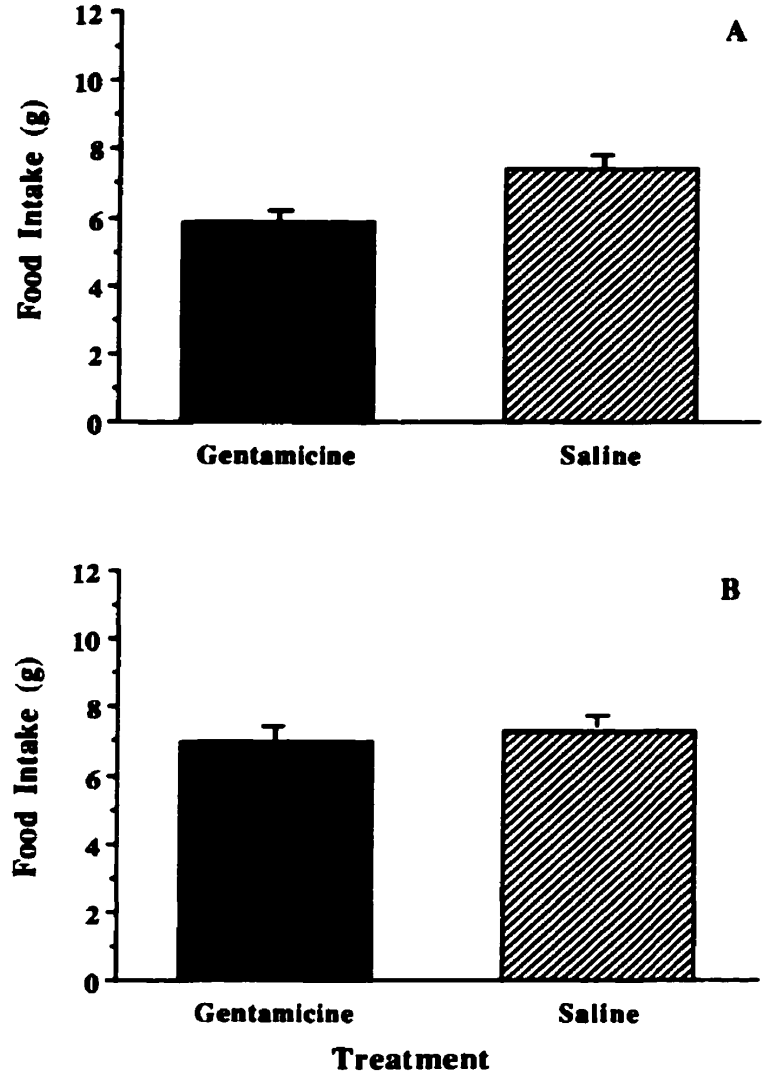


FIGURE A7b. The effect of treatment on food intake during the 14 h light phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 (B) with gentamicin (80 mg/kg, i.p.) or with saline. Data expressed as mean ± SEM.

TABLE A8. Repeated measures ANOVA of food intake (g) during the early, middle, and late parts of the dark phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(2,21)=3.753	0.0404					
	Modified ANOVA[5]	G-G			(8,168)=5.378	0.0005	(16,168)=2.155	0.0352	0.534
		H-F				0.0001		0.0177	0.751
Saline, 1300h	ANOVA		(2,10)=7.247	0.0113					
	Modified ANOVA	G-G			(8,80)=5.669	0.0018	(16,80)=5.184	0.0004	0.439
		H-F				0.0001		0.0001	0.842
Gentamicin, 0100h	ANOVA		(2,24)=4.839	0.0172					
	Modified ANOVA	G-G			(8,192)=7.976	0.0001	(16,192)=3.761	0.0002	0.638
		H-F				0.0001		0.0001	0.899
Saline, 0100h	ANOVA		(2,21)=4.249	0.0282					
	Modified ANOVA	G-G			(8,168)=5.620	0.0010	(16,168)=2.177	0.0477	0.427
		H-F				0.0002		0.0297	0.569

[1] Phase main effect (early, middle, and late parts of the dark phase)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

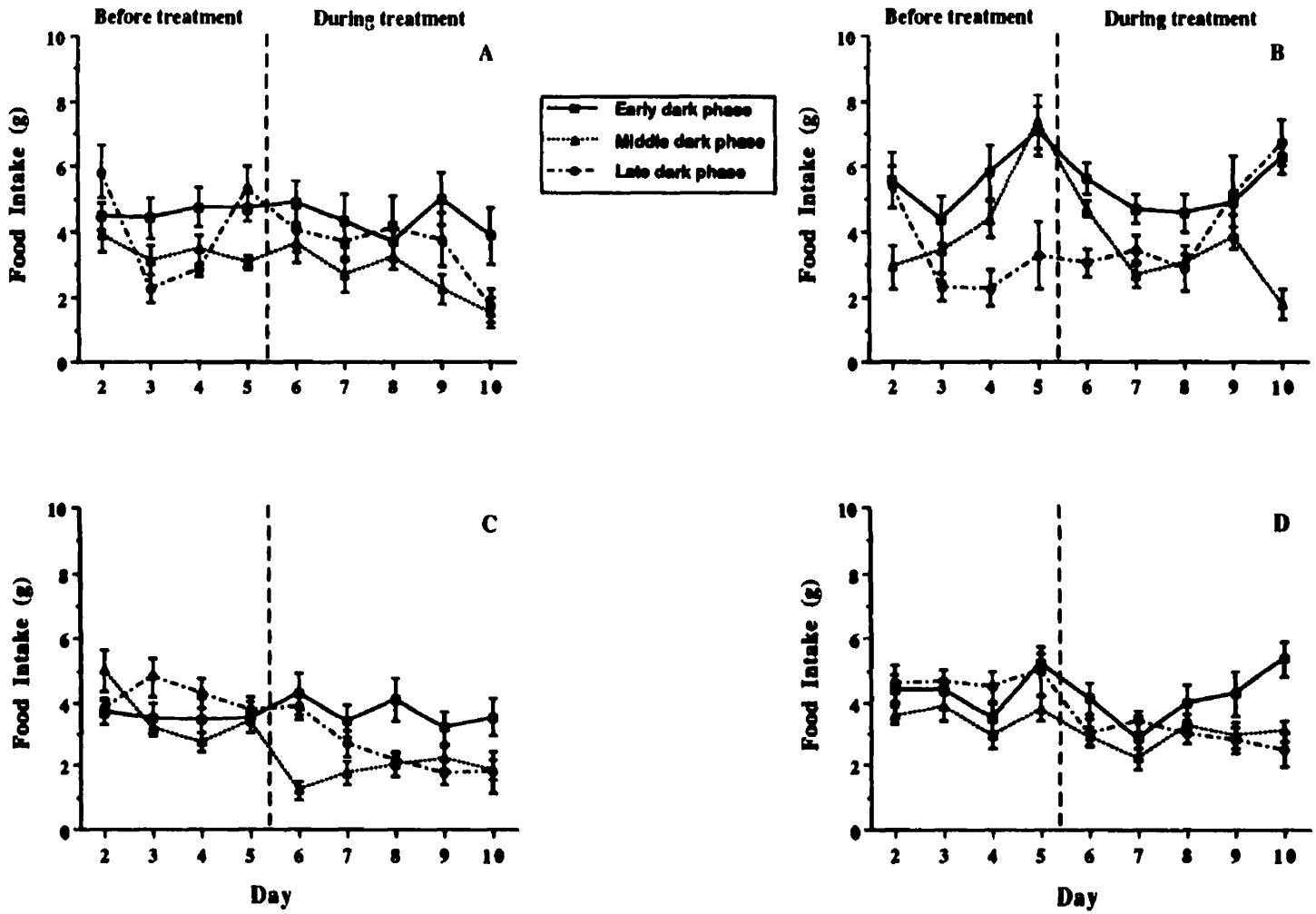


FIGURE A8a. The effect of day-by-phase on food intake during the early, middle and late parts of the dark phase of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See table Table A8 for significance.

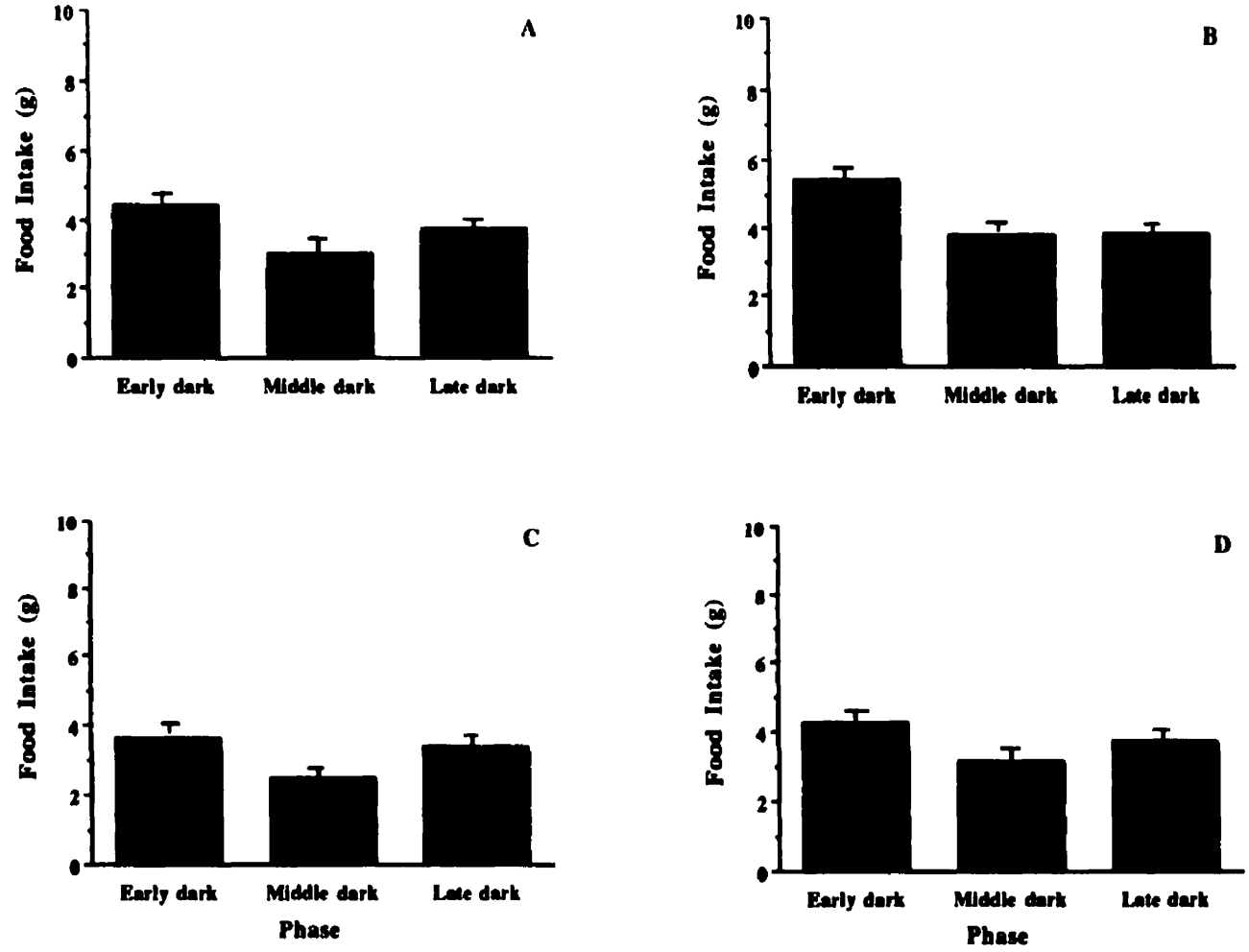


FIGURE A8b. The effect of the early, middle and late parts of the dark phase of the nycthemeral cycle on food intake in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. In graphics 'A', 'B', 'C', and 'D', since there was a significant interaction between phase and day, the difference among phases is not shown.

TABLE A9. Repeated measures ANOVA of food intake (g) during the early, middle, and late parts of the light phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(2,21)=3.154	0.0634					
	Modified ANOVA[5]	G-G			(8,168)=4.786	0.0025	(16,168)=2.621	0.0171	0.447
		H-F				0.0007		0.0078	0.601
Saline, 1300h	ANOVA		(2,19)=1.294	0.2973					
	Modified ANOVA	G-G			(8,152)=2.924	0.0491	(16,152)=5.712	0.0096	0.329
		H-F				0.0343		0.0043	0.427
Gentamicin, 0100h	ANOVA		(2,24)=15.896	0.0001					
	Modified ANOVA	G-G			(8,192)=2.073	0.0659	(16,192)=1.521	0.1307	0.691
		H-F				0.0405		0.0959	0.997
Saline, 0100h	ANOVA		(2,20)=34.611	0.0001					
	Modified ANOVA	G-G			(8,160)=4.945	0.0007	(16,160)=4.263	0.0001	0.575
		H-F				0.0001		0.0001	0.842

[1] Phase main effect (early, middle, and late parts of the light phase)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

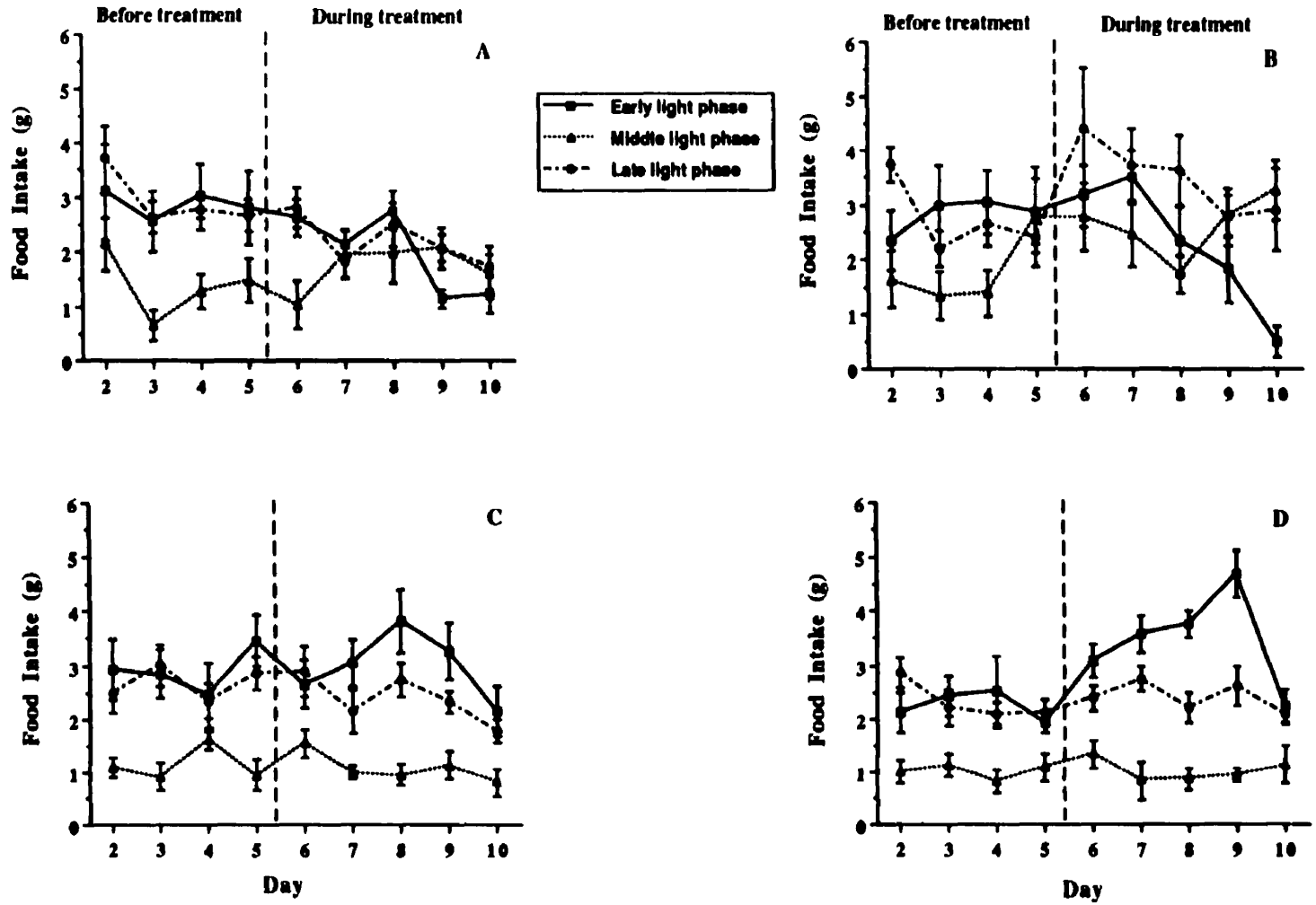


FIGURE A9a. The effect of day-by-phase on food intake during the early, middle and late parts of the light phase of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See table Table A9 for significance.

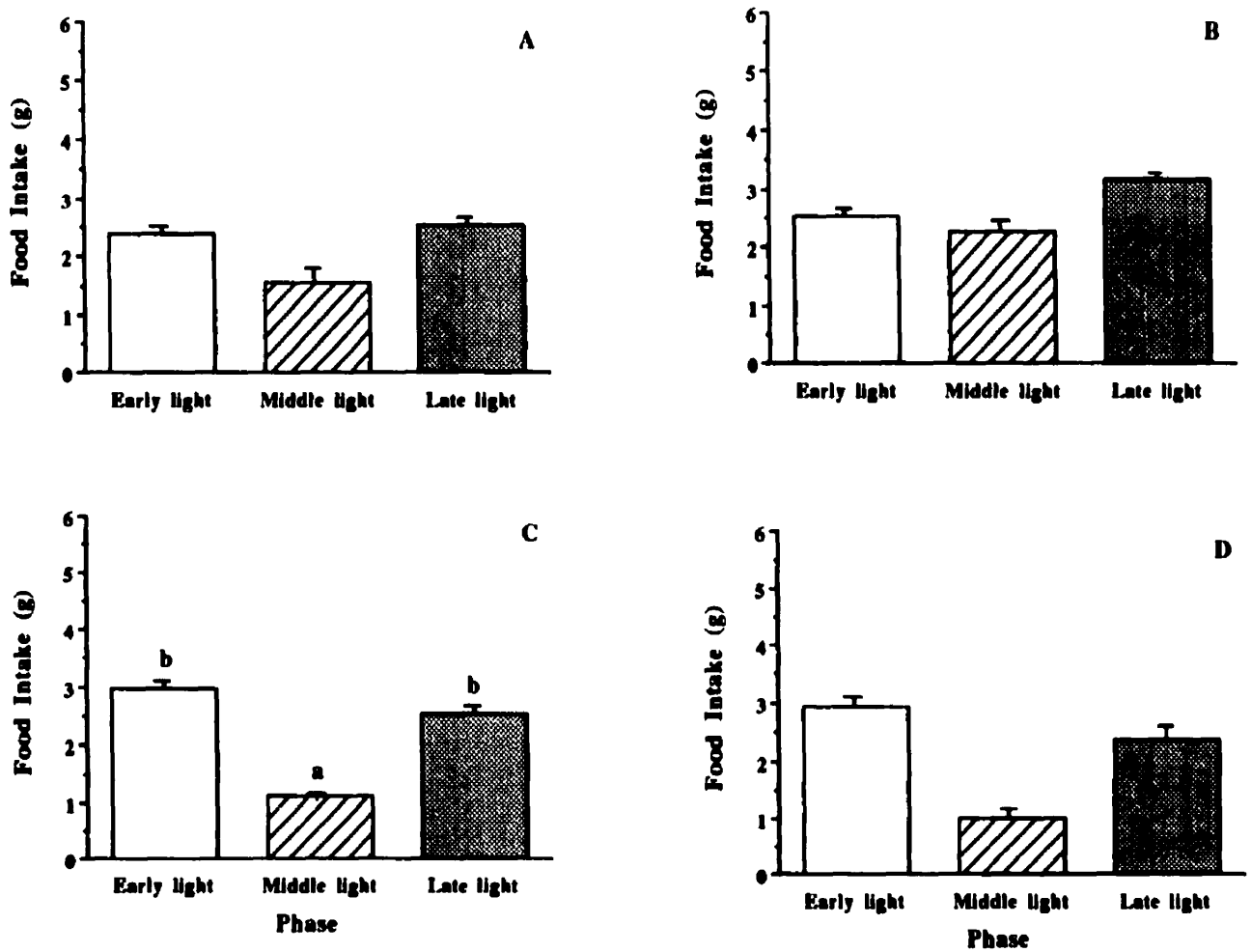


FIGURE A9b. The effect of the early, middle and late parts of the light phase of the nycthemeral cycle on food intake in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. Columns within a group not sharing the same lower case letter are significantly different from one another at $p<0.05$. In graphics 'A', 'B', and 'D', since there was a significant interaction between phase and day, the difference among phases in not shown.

TABLE A10. Repeated measures ANOVA of water intake (ml) over 24 h in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effect of day.

STATISTICAL ANALYSIS

Groups			Main Effect: Day[1] F value	Main Effect: Day[1] probability	Epsilon
Gentamicin, 1300h	Modified ANOVA[2]	G-G	(8,48)=8.692	0.5009	0.410
		H-F		0.5751	0.965
Saline, 1300h	Modified ANOVA	G-G	(8,48)=2.634	0.1036	0.282
		H-F		0.0654	0.460
Gentamicin, 0100h	Modified ANOVA	G-G	(8,48)=0.557	0.6514	0.378
		H-F		0.7726	0.803
Saline, 0100h	Modified ANOVA	G-G	(8,40)=1.619	0.2339	0.329
		H-F		0.1787	0.729

[1] Day main effect (days 2 to 10)

[2] The within-subject effect (i.e., day main effect) is tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

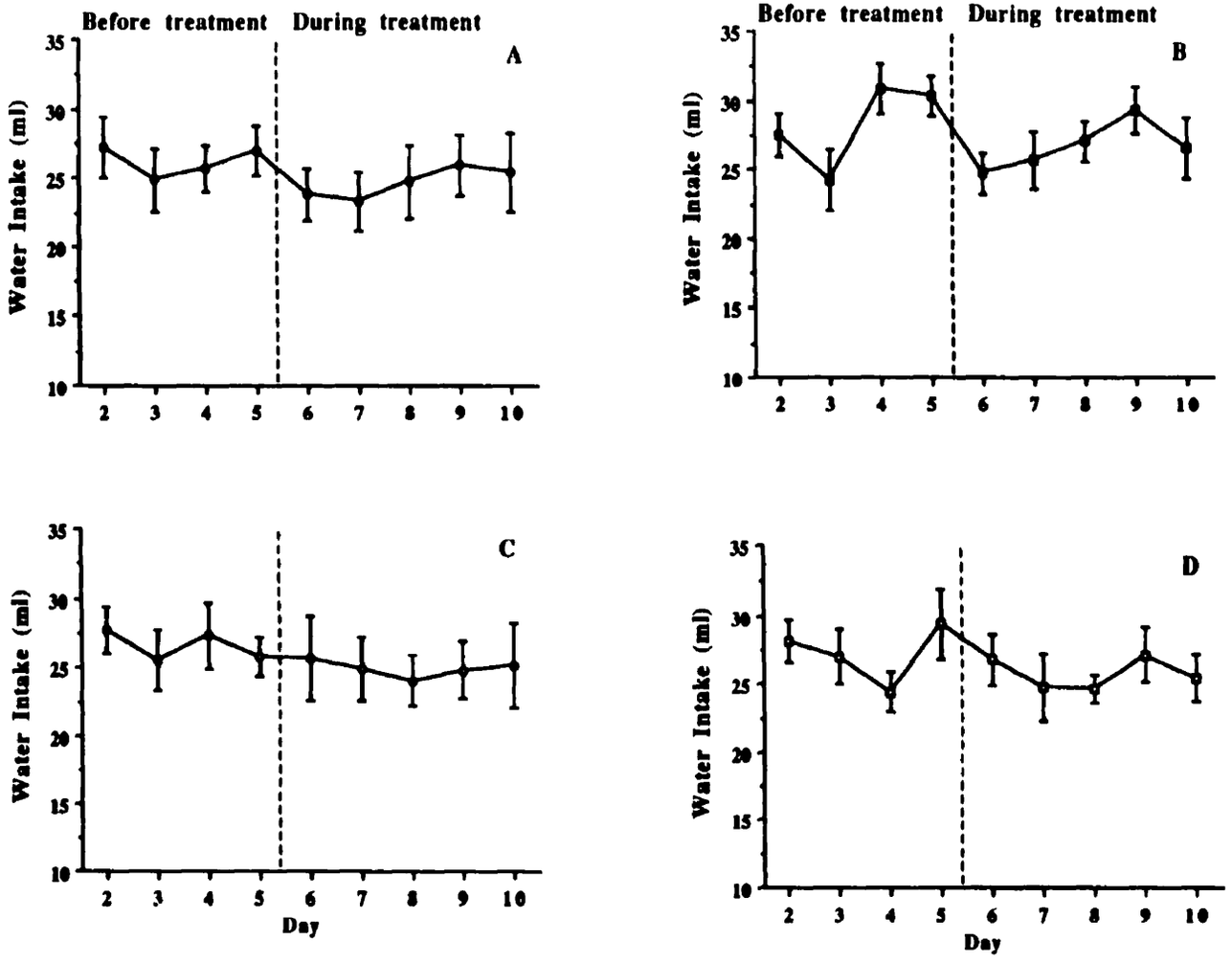


FIGURE A10a. The effect of day on water intake over 24 h in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See Table A10 for significance.

TABLE A11. Repeated measures ANOVA of water intake (ml) over 24 h in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,13)=1.472	0.2466					
	Modified ANOVA[5]	G-G			(4,52)=1.418	0.2564	(4,52)=0.166	0.8937	0.639
		H-F				0.2470		0.9391	0.871
0100h	ANOVA		(1,14)=0.276	0.6076					
	Modified ANOVA	G-G			(4,56)=1.173	0.3322	(4,56)=0.292	0.8547	0.855
		H-F				0.8817		0.8817	1.245

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

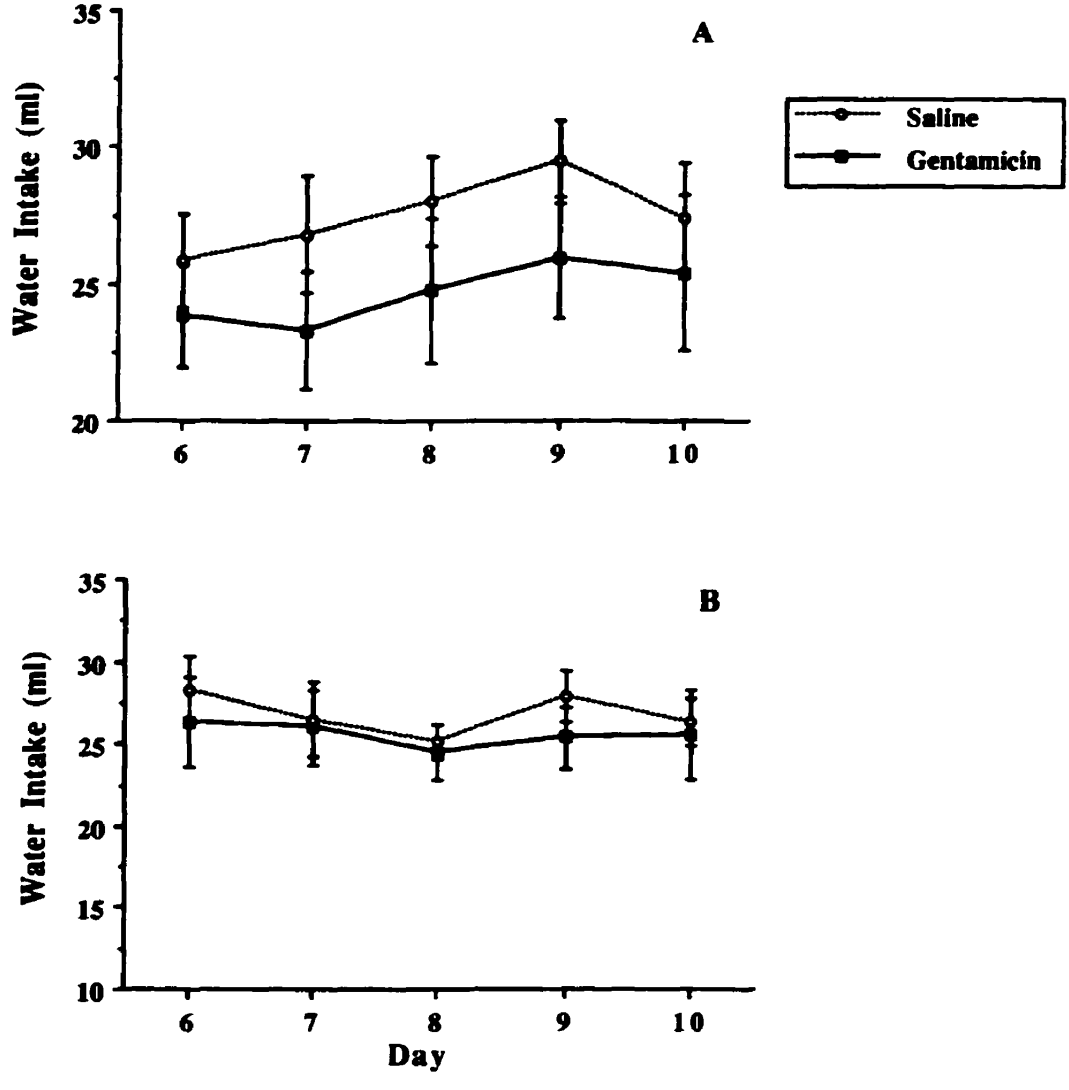


FIGURE A11a. The effect of day-by-treatment on water intake over 24 h in rats treated at 1300 h or at 0100 h with gentamicine (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See table A11 for significance.

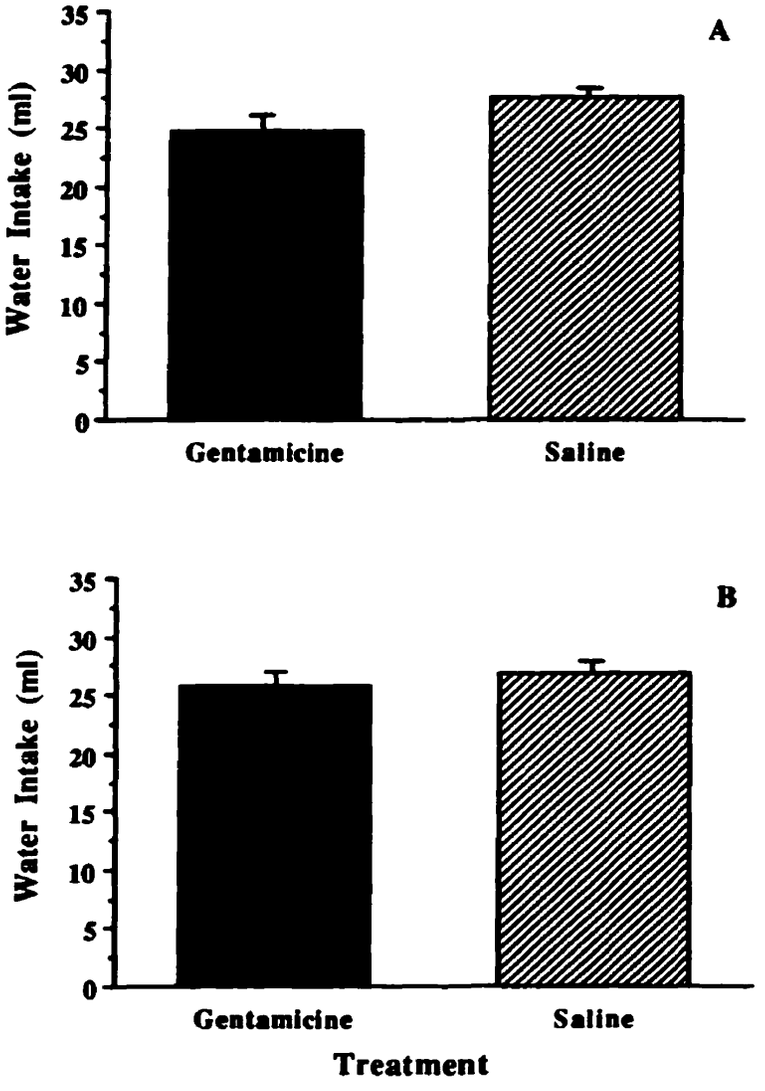


FIGURE A11b. The effect of treatment on the 24 h water intake in rats treated at 1300 h (A) or at 0100 h (B) with gentamicine (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM.

TABLE A12. Repeated measures ANOVA of water intake (ml) during the 14 h light and 10 h dark phases of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(1,12)=32.558	0.0001					
	Modified ANOVA[5]	G-G			(8,96)=0.721	0.6006	(8,96)=2.249	0.0663	0.580
		H-F				0.6723		0.0301	1.070
Saline, 1300h	ANOVA		(1,12)=101.796	0.0001					
	Modified ANOVA	G-G			(8,96)=2.823	0.0364	(8,96)=1.724	0.1621	0.487
		H-F				0.0132		0.1210	0.811
Gentamicin, 0100h	ANOVA		(1,8)=43.164	0.0001					
	Modified ANOVA	G-G			(8,96)=0.567	0.6637	(8,96)=0.770	0.5343	0.433
		H-F				0.7388		0.5851	0.681
Saline, 0100h	ANOVA		(1,10)=102.417	0.0001					
	Modified ANOVA	G-G			(8,80)=1.858	0.1379	(8,80)=2.734	0.0430	0.493
		H-F				0.0841		0.0122	0.935

[1] Phase main effect (14 h light and 10 h dark phases)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

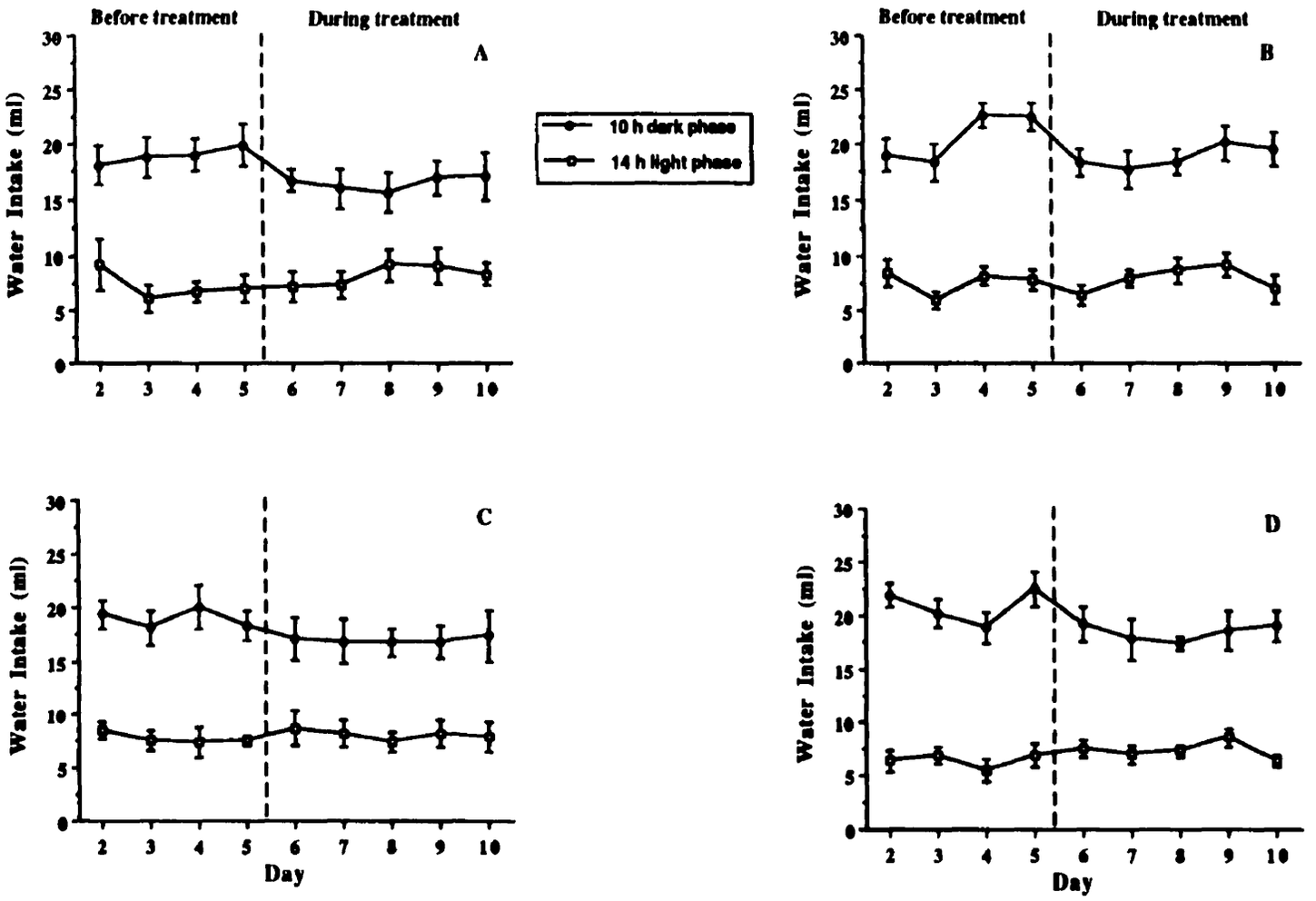


FIGURE A12a. The effect of day-by-phase on water intake during the 14 h light and 10 h dark phases of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicine (C) or saline (D). Data expressed as mean ± SEM. See table A12 for significance.

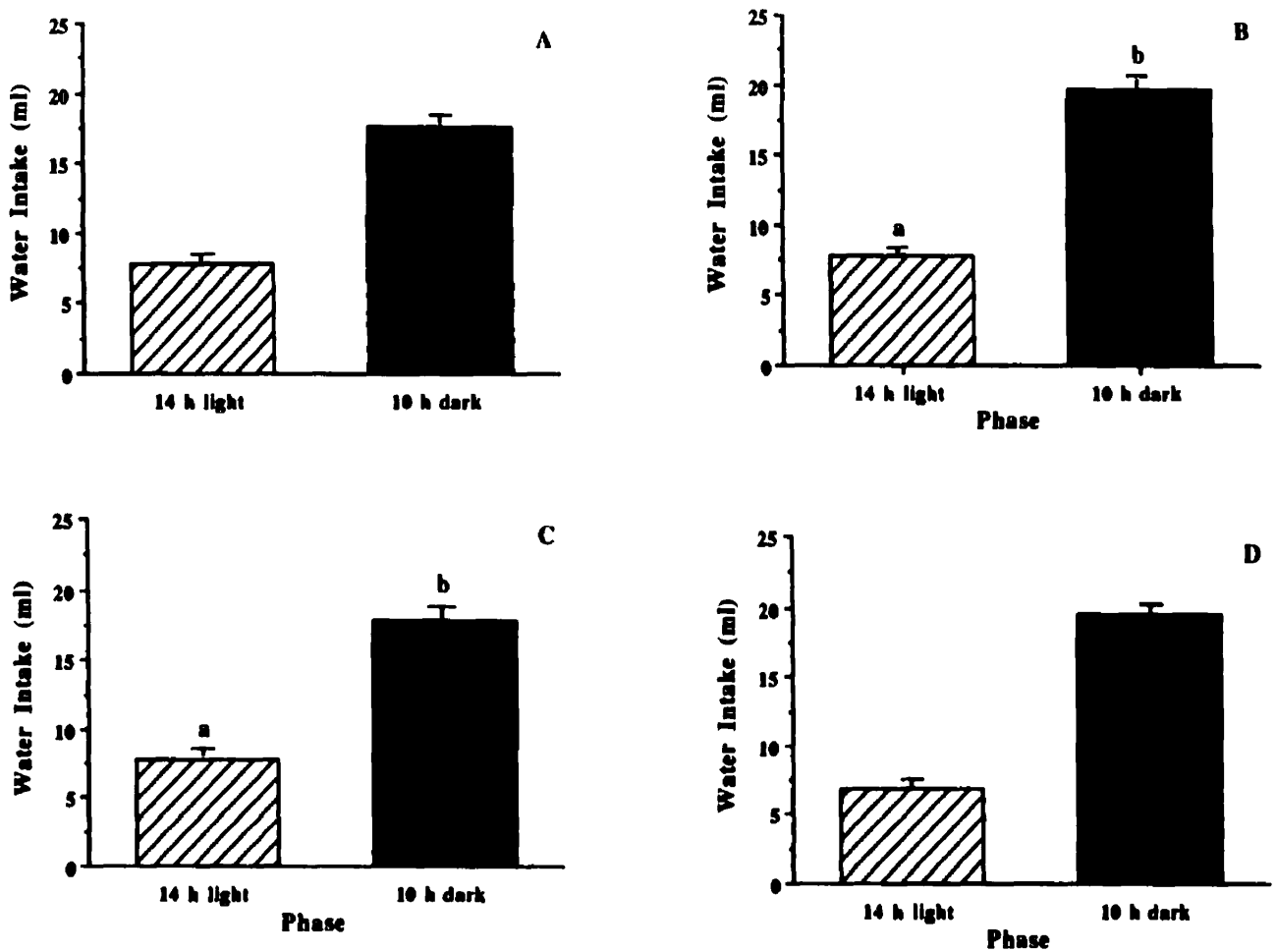


FIGURE A12b. The effect of the 14 h light and 10 h dark phases of the nycthemeral cycle on water intake in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. Columns within a group not sharing the same lower case letter are significantly different from one another at $p<0.05$. In graphics 'A' and 'D', since there was a significant interaction between phase and day, the difference between phases is not shown.

TABLE A13. Repeated measures ANOVA of water intake (ml) during the 10 h dark phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,13)=2.997	0.1070					
	Modified ANOVA[5]	G-G			(4,52)=0.819	0.4876	(4,52)=0.091	0.9609	0.722
		H-F				0.5190		0.9849	1.022
0100h	ANOVA		(1,14)=0.964	0.3427					
	Modified ANOVA	G-G			(4,56)=1.150	0.3401	(4,56)=0.208	0.8924	0.758
		H-F				0.3425		0.9332	1.061

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

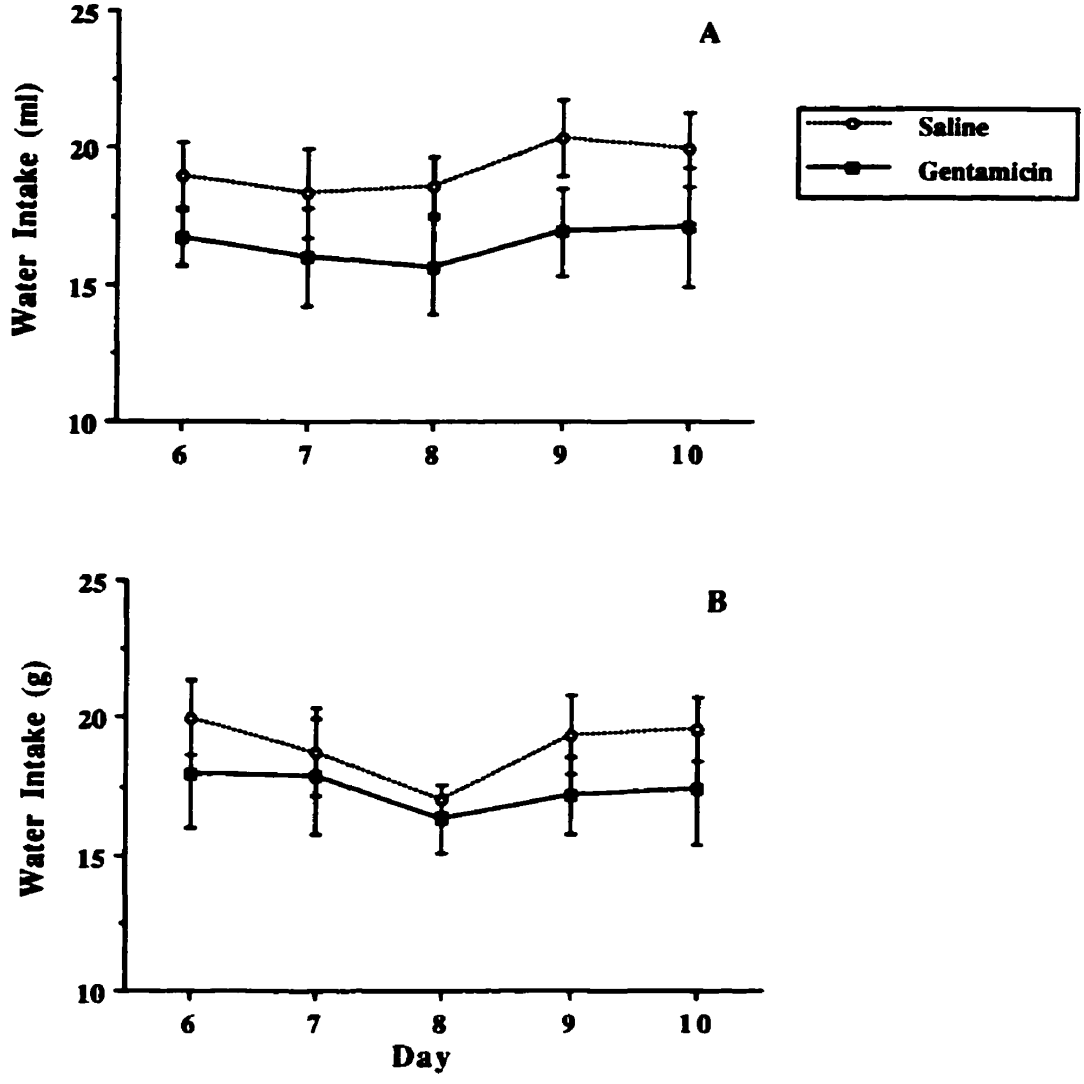


FIGURE 13a. The effect of day-by-treatment on water intake during the 10 h dark phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 h (B) with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See table A13 for significance.

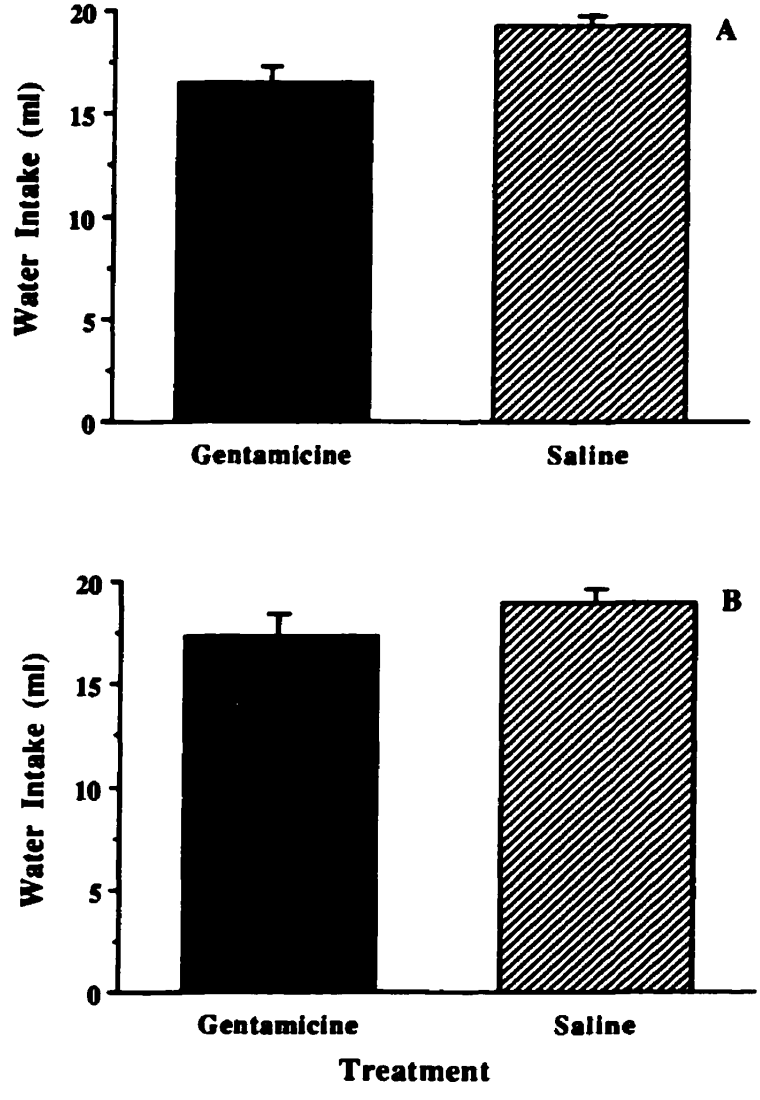


FIGURE A13b. The effect of treatment on water intake during the 10 h dark phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 h (B) with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM.

TABLE A14. Repeated measures ANOVA of water intake (ml) during the 14 h light phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of treatment, day, and day-by-treatment.

STATISTICAL ANALYSIS

			Main Effects						
			Treatment[1]		Day[2]		Day*Treatment[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
1300h	ANOVA[4]		(1,13)=0.003	0.9576					
	Modified ANOVA[5]	G-G			(4,52)=3.755	0.1680	(4,52)=0.595	0.6287	0.783
		H-F				0.0093		0.6677	1.139
0100h	ANOVA		(1,14)=0.067	0.7966					
	Modified ANOVA	G-G			(4,56)=0.590	0.6580	(4,56)=0.487	0.7299	0.920
		H-F				0.6716		0.7450	1.377

[1] Treatment main effect (gentamicin and saline)

[2] Day main effect (days 6 to 10)

[3] Day-by-treatment interaction

[4] ANOVA is restricted to the between-subject effect (i.e., treatment); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-treatment interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

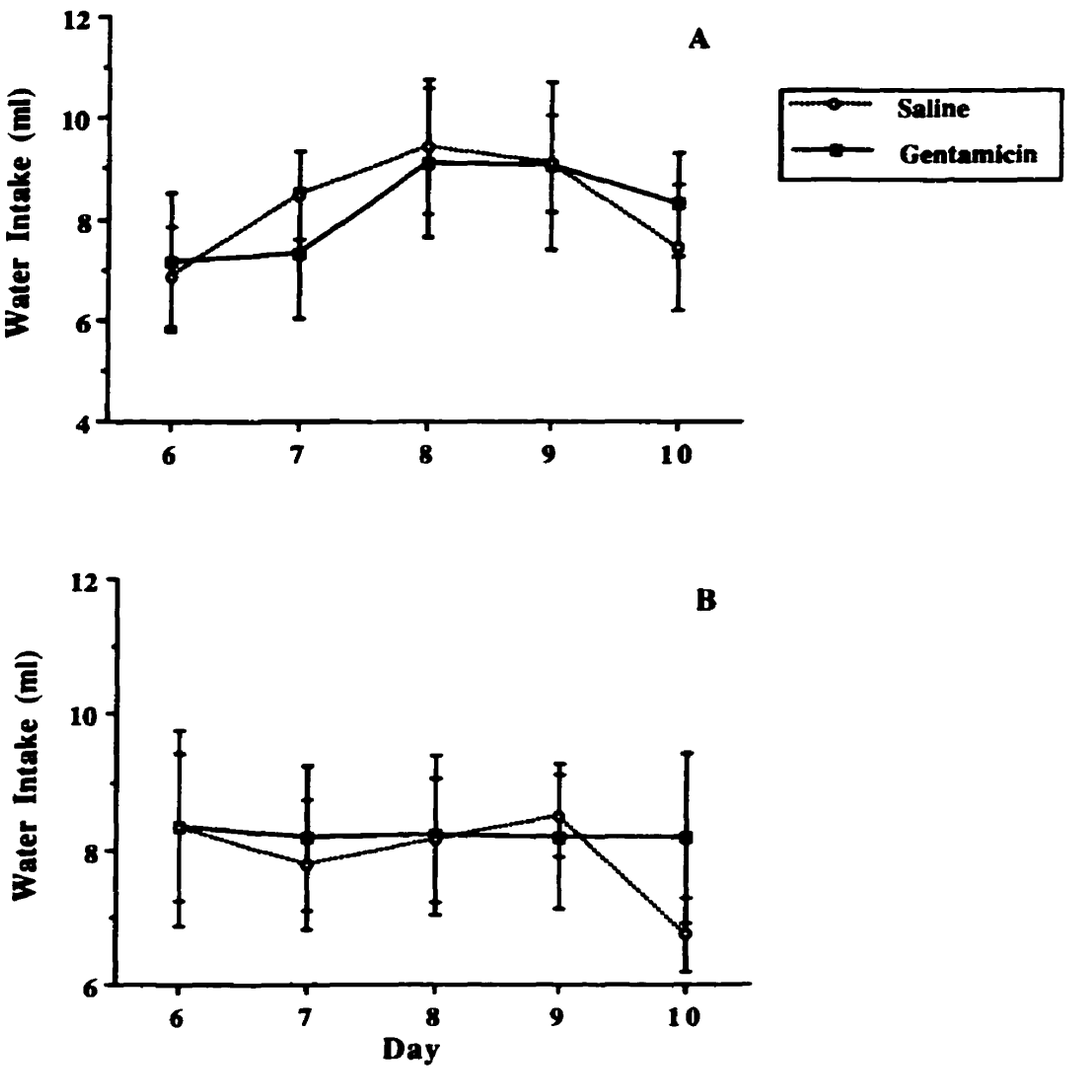


FIGURE A14a. The effect of day-by-treatment on water intake during the 14 h light phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100h (B) with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See table A14 for significance.

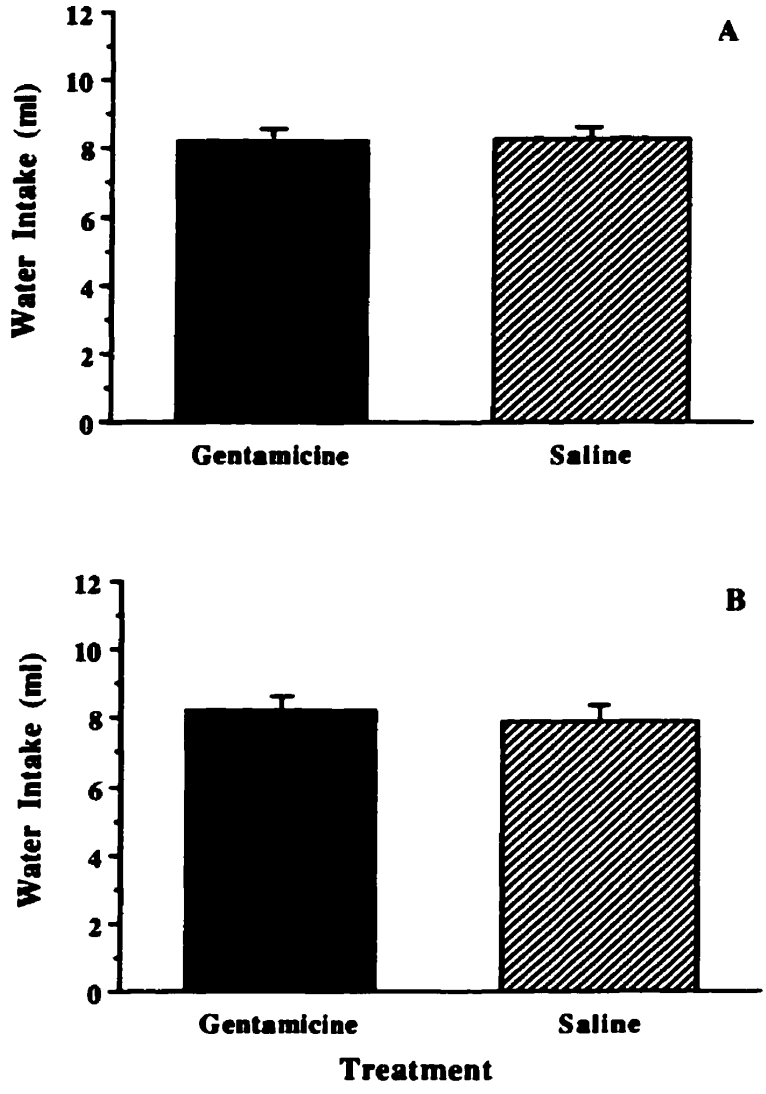


FIGURE A14b. The effect of treatment on water intake during the 14 h light phase of the nycthemeral cycle in rats treated at 1300 h (A) or at 0100 h (B) with gentamicin (80 mg/kg, i.p.) or saline. Data expressed as mean ± SEM.

TABLE A15. Repeated measures ANOVA of water intake (ml) during the early, middle, and late parts of the dark phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(2,18)=6.473	0.0076					
	Modified ANOVA[5]	G-G			(8,144)=2.282	0.0641	(16,144)=3.223	0.0027	0.533
		H-F				0.0373		0.0004	0.798
Saline, 1300h	ANOVA		(2,18)=5.894	0.0107					
	Modified ANOVA	G-G			(8,144)=5.161	0.0006	(16,144)=5.524	0.0001	0.560
		H-F				0.0001		0.0001	0.852
Gentamicin, 0100h	ANOVA		(2,18)=4.546	0.0252					
	Modified ANOVA	G-G			(8,144)=0.769	0.5717	(16,144)=3.182	0.0020	0.577
		H-F				0.6237		0.0002	0.887
Saline, 0100h	ANOVA		(2,15)=7.401	0.0058					
	Modified ANOVA	G-G			(8,120)=2.180	0.0754	(16,120)=1.734	0.1009	0.546
		H-F				0.0399		0.0571	0.901

[1] Phase main effect (early, middle, and late parts of the dark phase)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

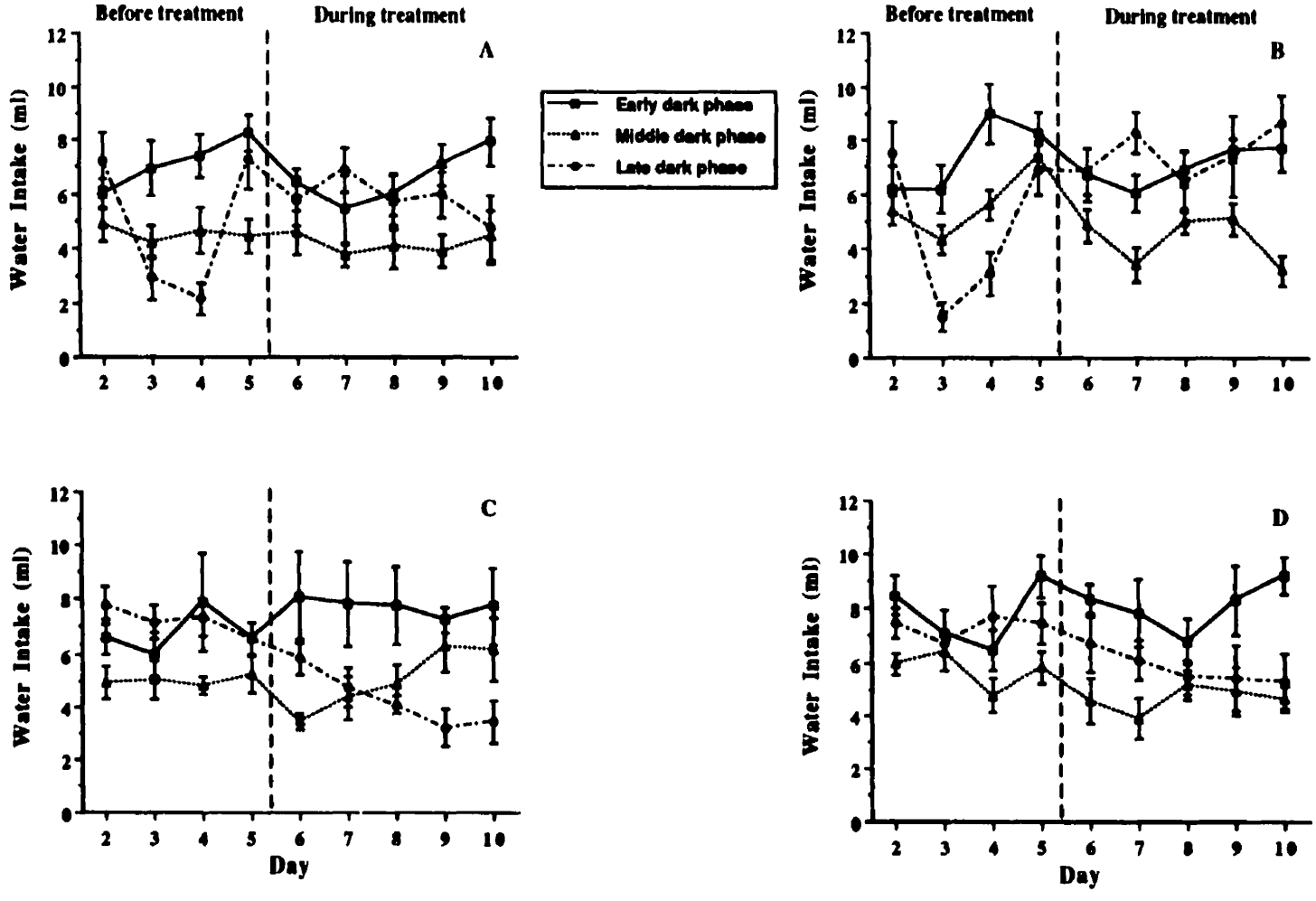


FIGURE A15a. The effect of day-by-phase on water intake during the early, middle and late parts of the dark phases of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See table A15 for significance.

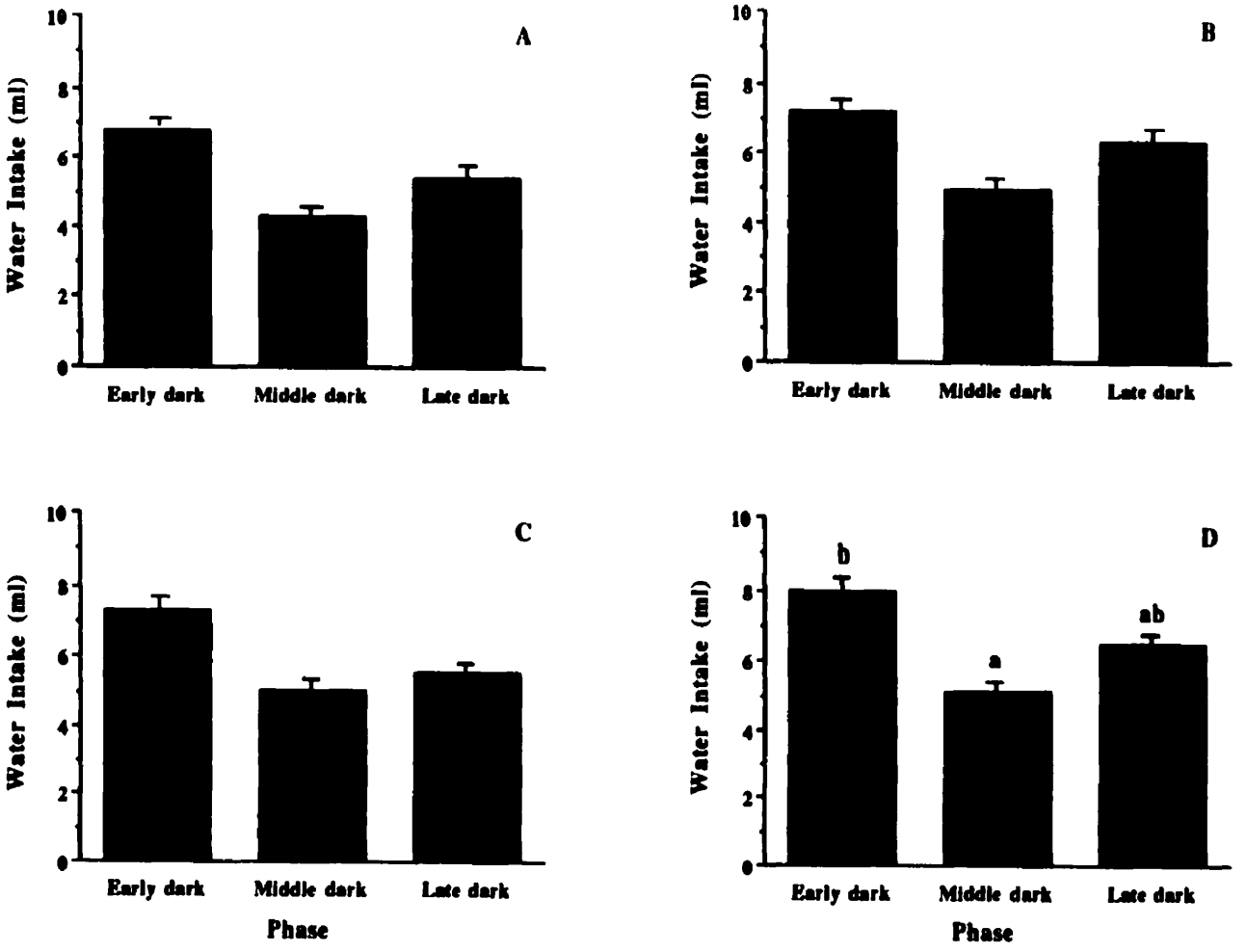


FIGURE A15b. The effect of the early, middle and late parts of the dark phase of the nycthemeral cycle on water intake in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. Columns within a group not sharing the same lower case letter are significantly different from one another at $p<0.05$. In graphics 'A' 'B'and 'C', since there was a significant interaction between phase and day, the difference among phases is not shown.

TABLE A16. Repeated measures ANOVA of water intake (ml) during the early, middle, and late parts of the light phase of the nycthemeral cycle in rats treated with gentamicin (80 mg/kg, i.p.) or saline at 0100 h or 1300 h: the effects of phase, day, and day-by-phase.

STATISTICAL ANALYSIS

			Main Effects						
			Phase[1]		Day[2]		Day*Phase[3]		Epsilon
Groups			F value	probability	F value	probability	F value	probability	
Gentamicin, 1300h	ANOVA[4]		(2,18)=0.483	0.6247					
	Modified ANOVA[5]	G-G			(8,144)=2.043	0.0852	(16,144)=6.164	0.0001	0.585
		H-F				0.0523		0.0001	0.904
Saline, 1300h	ANOVA		(2,18)=1.902	0.1781					
	Modified ANOVA	G-G			(8,144)=1.542	0.1892	(16,144)=3.088	0.0026	0.586
		H-F				0.1562		0.0003	0.906
Gentamicin, 0100h	ANOVA		(2,18)=12.937	0.0003					
	Modified ANOVA	G-G			(8,144)=0.226	0.9386	(16,144)=0.959	0.4797	0.564
		H-F				0.9774		0.4977	0.86
Saline, 0100h	ANOVA		(2,15)=40.083	0.0001					
	Modified ANOVA	G-G			(8,120)=1.205	0.3167	(16,120)=2.880	0.0060	0.567
		H-F				0.3037		0.0007	0.951

[1] Phase main effect (early, middle, and late parts of the light phase)

[2] Day main effect (days 2 to 10)

[3] Day-by-phase interaction

[4] ANOVA is restricted to the between-subject effect (i.e., phase); a rat is a "subject".

[5] The within-subject effects (i.e., day main effect, day-by-phase interaction) are tested in the modified ANOVA. In this modified testing procedure, the probabilities of significance P>F are adjusted by using the Greenhouse Geisser's (1959) and Huynh and Feldt's (1976) estimates of Box's (1954a, b) epsilon correction factor. The correction is applied to the number of degrees of freedom of the ANOVA F test statistic in order to take into account the autocorrelation and heteroscedasticity of the variables over days; the lower the epsilon value, the stronger the required correction due to autocorrelation and heteroscedasticity. The corresponding adjusted probabilities are denoted G-G and H-F, respectively.

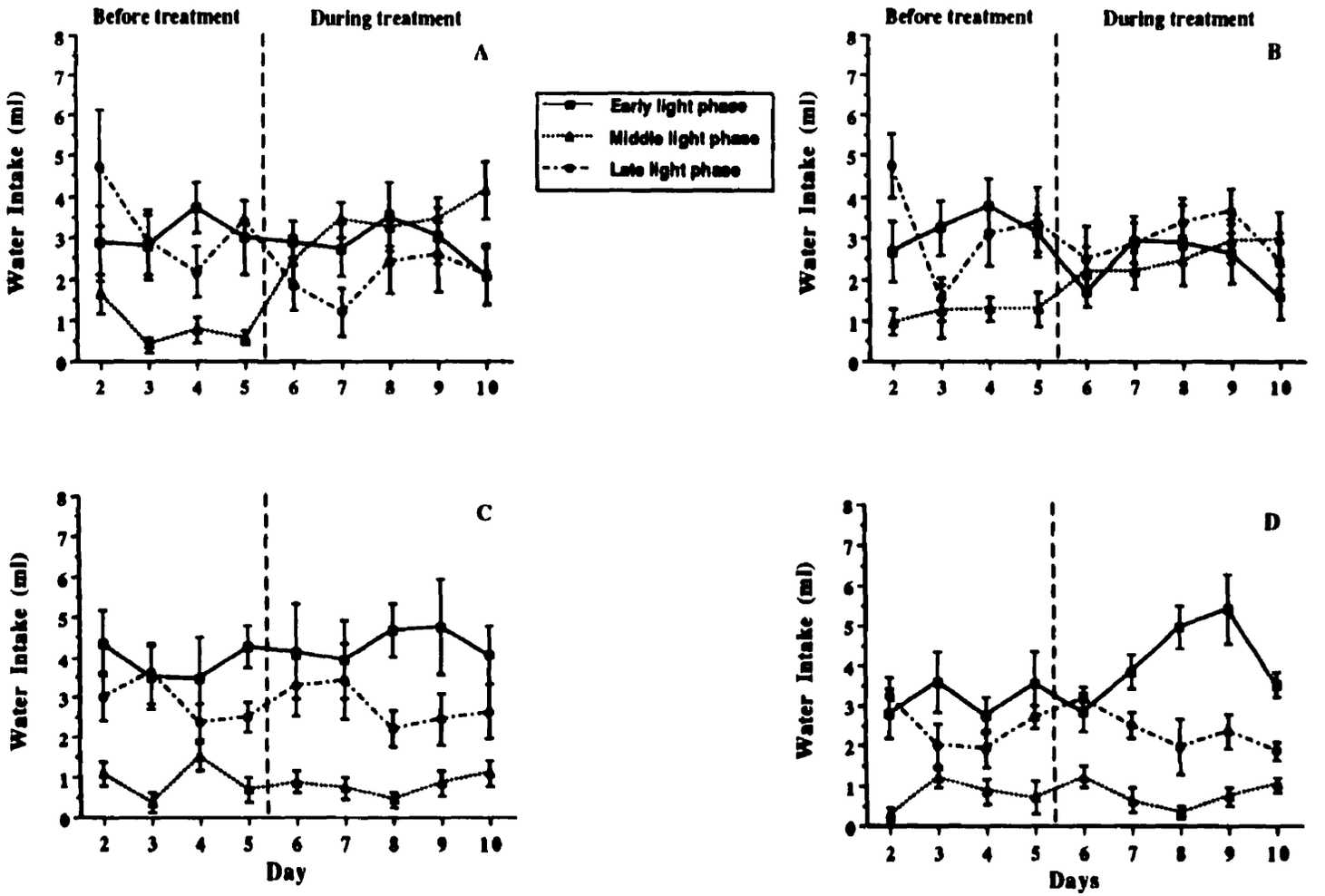


FIGURE A16a. The effect of day-by-phase on water intake during the early, middle and late parts of the light phases of the nycthemeral cycle in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. See table A16 for significance.

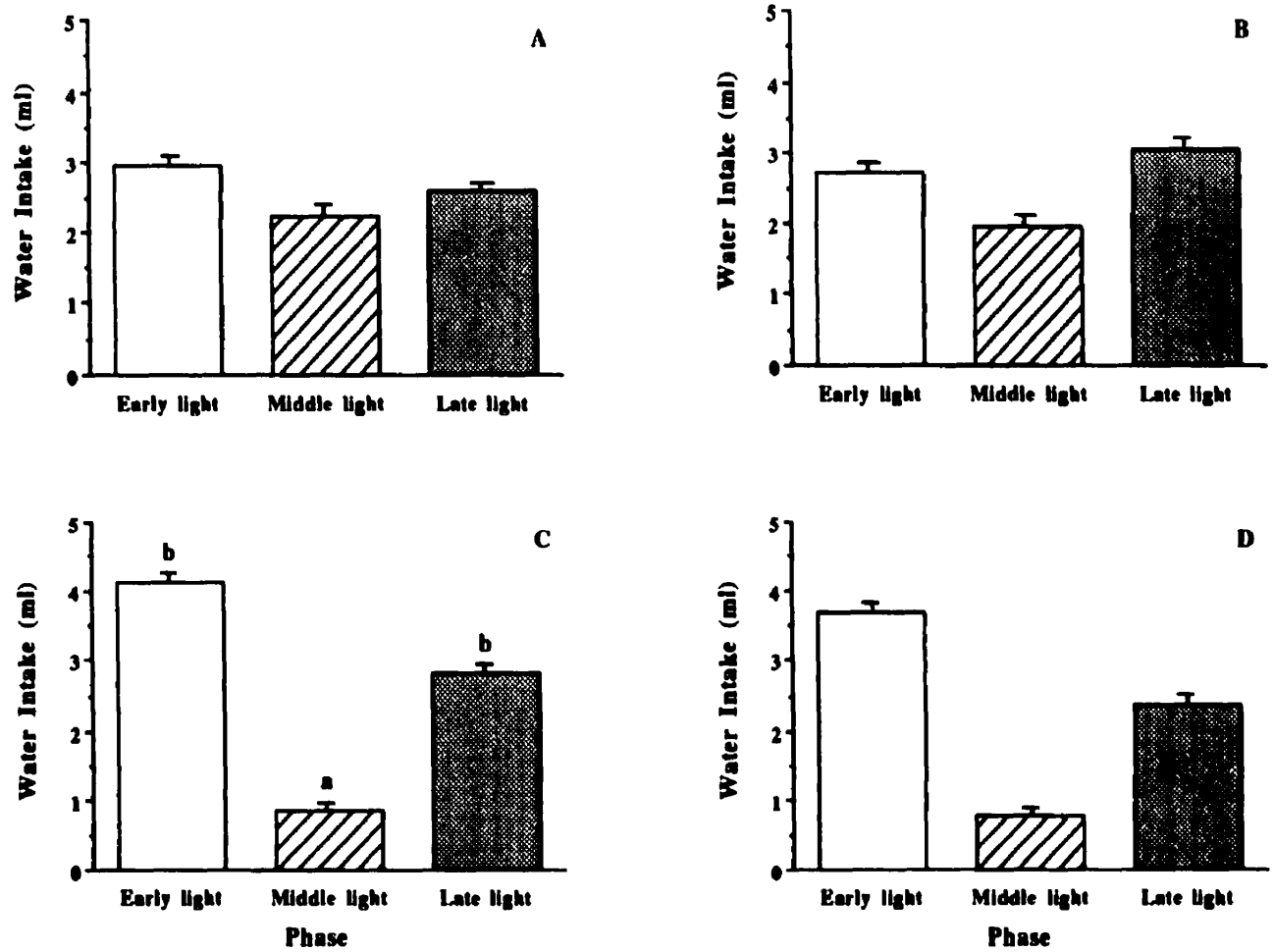


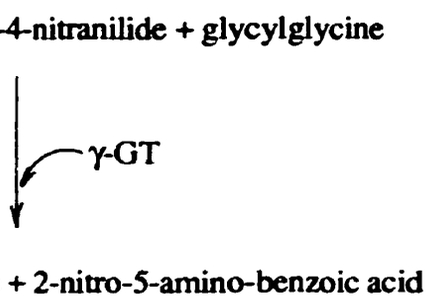
FIGURE A16b. The effect of the early, middle and late parts of the light phase of the nycthemeral cycle on water intake in rats treated at 1300 h with gentamicin (80 mg/kg, i.p.) (A) or saline (B), or at 0100 h with gentamicin (C) or saline (D). Data expressed as mean ± SEM. Columns within a group not sharing the same lower case letter are significantly different from one another at $p<0.05$. In graphics 'D', since there was a significant interaction among phase and day, the difference between phases is not shown.

PROTOCOL FOR NEPHROTOXICITY MEASUREMENTS

1. Urinary enzymes activity

1.1 Urinary γ-glutamyltranspeptidase

Principle: γ-glutamyltranspeptidase (γ-GT) is a brush-border enzyme enabling the amino acids to enter the cells. It is related to the state of the brush-border membrane of proximal tubules in the renal cortex. An increase in the concentration of this enzyme in the urine indicates a deterioration of the brush-border membrane. The determination of γ-GT activity is performed using the method developed by Persijn and van der Slik (1976). Briefly, the enzyme substrate L- γ -glutamyl-3-carboxy-4-nitranilide is mixed with the buffer glycylglycine. This buffer-substrate solution is then added to the urine. γ-GT, if present in the urine, will transform the buffer-substrate into L-glycylglycine and 2-nitro-5-amino-benzoic acid. γ-GT is then measured by a spectrophotometric method by using the absorbance of the degration product 2-nitro-5-amino-benzoic acid at a wavelenght of 405 nm.



Material: Trizma base (tris [hydroxymethyl] aminomethane)

Glycylglycine (Gly-Gly)

L-y-glutamyl-3-carboxy-4-nitranilide

Fresh urine samples (24h urines)

Reagent Preparation:

A. Tris-glycylglycine buffer: tris 0.1 M, glycylglycine 0.1 M

-dissolve 1.21 g of trizma base and 1.32 g of glycylglycine in 100 ml distilled water

-conservation time: 1 month at 4°C

B. Buffered substrate solution: L-γ-glutamyl-3-carboxy-4-nitranilide

-dissolve 0.01385 g of L-γ-glutamyl-3-carboxy-4-nitranilide in 8 ml of tris-glycylglycine buffer

-adjust the pH to 8.25 by adding NaOH 1.0 N (2 to 4 drops) and make up the volume to 10 ml

-conservation time: 24h at 4°C

Procedure:

-put 800 µl of buffered substrate in a cuvet

-add 200 µl of urine

-with a spectrophotometer, read at 0, 1, 2, 3, and 4 minutes at 405 nm against the blank (empty well).

-temperature: 25°C

Calculation of Activity:

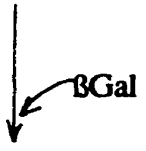
Enzyme activity (as International Units per liter [U/l]): mean variation between each minute * 1158

Enzyme output (mU/24 h): U/l * 24h urine volume

1.2 Urinary ß-galactosidase

Principle: ß-galactosidase (ßGal) is found in the lysosomes of proximal tubular cells. Its presence in the urine is an indication of toxicity. The determination of ßGal activity was performed using the colorimetric method of Maruhn (1976). ßGal is measured by spectrophotometric method by using the absorbance of the degradation product 4-nitrophenol at 405 nm.

4-nitrophenyl-ß-D-galactopyranoside



4-nitrophenol + ß-D-galactopyranoside

Material: Citric acid, anhydrous, powder

Citrate trisodium, dihydrate, granular

Para-nitrophenyl-ß-D-galactopyranoside

2-amino-2-methyl-propan-1-ol

Fresh urine samples (24h urines)

Reagents Preparation:

A. Citrate buffer, 0.1 M, pH 4.00 at 37°C

-dissolve 3.82 g of citrate andydre in 200 ml distilled water

-dissolve 5.88 g of trisodium dihydrate in 200 ml distilled water

- heat these solutions to 37°C in a water bath

-slowly incorporate the 200 ml of citrate andyde to citrate trisodium dihydrate

-conservation time: 3 months at 4°C

B. Buffered substrate solution: PNPG 5 mM/l

-dissolve 0.11 g of para-nitrophenyl-ß-D-galactopyranoside (PNPG) in 75 ml of buffer A

-dispensed 0.2 ml of this solution in disposible glass tube (12X75). Close the tubes and freeze at -25°C

C. 2-amino-2-methyl-propan-1-ol (AMP buffer)

-dissolve 6.6855 g of liquid AMP in 50 ml distilled water. At 37°C, adjust the pH to 10.25 with HCl 8N and make up the volume to 100 ml with distilled water

-conservation time: 3 months at 4°C

D. HCl 8 N

6 ml of HCl 12 N for 3 ml of distilled water

Procedure:

-prewarm urines at 37°C in a water bath

-thaw and prewarm tubes containing the buffer-substrate solution to 37°C. The number of tubes corresponding to the number of urine samples plus one. To the extra tube, add 200 µl of distilled water. This tube is the blank of the experiment. If its absorbance value exceed 0.07, the solutions should be discarded.

-distribute 200 µl of citrate buffer in disposible tubes (12 X 75)

-add 200 µl of prewarmed urines to the buffer-substrate and citrate buffer containing tubes

-15 minutes later, terminate the reaction by adding 200 µl of AMP buffer to all tubes

-read the contents of all tubes immediatly at 405 nm against the blank reagent

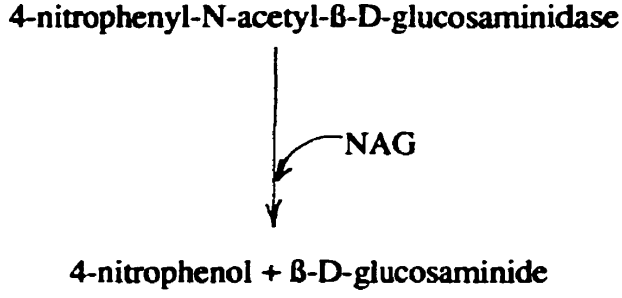
Calculation of Activity

-Enzyme activity (U/l): difference between the tubes of buffer-substrate and citrate buffer * 10.811

-Enzyme output (mU/24 h): U/l * diuresis (ml/24 h)

1.3 Urinary N-acetyl-ß-D-glucosaminidase

Principle: N-acetyl-ß-D-glucosaminidase (NAG) is found in the lysosomes of proximal tubular cells. Its presence in the urine is an indication of toxicity. The determination of NAG activity was performed using the colorimetric method of Maruhn (1976). NAG was measured by a spectrophotometric method by using the absorbance of the degradation product, 4-nitrophenol at 405 nm.



Material: Citric acid, anhydrous, powder

Citrate trisodium, dihydrate, granular

4-nitrophenyl N-acetyl-ß-D-glucosaminide

2-amino-2-methyl-propan-1-ol

Fresh urine samples

Reagents Preparation:

A. Citrate buffer, 0.1 M, pH 4.15 at 37°C

-dissolve 3.82 g of citrate andydre in 200 ml distilled water

-dissolve 5.88 g of trisodium dihydrate in 200 ml distilled water

- heat these solutions to 37°C

-slowly incorporate the 200 ml of citrate andyde to citrate trisodium dihydrate

-conservation time: 3 months at 4°C

B. Buffered substrate solution: PNAG 10 mM/l

-dissolve 0.34 g of para-nitrophenyl-N-acetyl-ß-D-glucosaminidase in 100 ml of citrate buffer

-dispense 0.2 ml of this solution in disposible glass tube (12X75). Close the tubes and freeze at -25°C

-conservation time: 4 months at -25°C

C. 2-amino-2-methyl-propan-1-ol buffer (AMP buffer)

-dissolve 6.69 g of liquid AMP in 50 ml distilled water. At 37°C, adjust the pH to 10.25 with HCl 8N and make up the volume to 100 ml with distilled water

-conservation time: 3 months at 4°C

D. HCl 8 N

6 ml of HCl 12 N for 3 ml of distilled water

Procedure:

-prewarm urines at 37°C

-thaw and prewarm tubes containing the buffer-substrate solution to 37°C. The number of tubes corresponding to the number of urine samples plus one. To the extra tube, add 200 µl of distilled water. This tube is the blank of the experiment; if its absorbance value exceed 0.07, the solutions should be discarded.

-distribute 200 µl of citrate buffer in disposible tubes (12 X 75)

-add 200 µl of prewarmed urines to the buffer-substrate and citrate buffer containing tubes

-15 minutes later, terminate the reaction by adding 200 µl of AMP buffer to all tubes

-read the contents of all tubes immediatly at 405 nm against the reagent blank

Calculation of Activity

-Enzyme activity (U/l): difference between the tubes of buffer-substrate and citrate buffer * 10.811

-Enzyme output (mU/24 h): U/l * diuresis (ml/24 h)

2. Gentamicin level in renal cortex

Homogenate preparation:

-weigh the cortices and put them in cold distilled water

-homogenize with tissue tearor and sonicate for 10 to 15 seconds (sonicate just before the measurement preferably)

-dilute in TDX buffer

-use 5 ml tubes with stoppers

Verication of TDX system:

-put 75 µl of each of the three controls (L,M,H) in three tubes

-read

-the controls should be between: 0.85 and 1.15 µg/ml for L
3.60 and 4.40 µg/ml for M
7.20 and 8.80 µg/ml for H

-if the controls are unacceptable, the TDX must be recalibrated

Calculation:

$$\text{Gentamicin} = \frac{2\text{ ml} + \text{cortex weight}}{\text{cortex weight}} * \text{sample concentration} * \text{dilution factor}$$

3. [^{3}H]-thymidine incorporation into DNA

Day 1: preparation of material and solutions

Day 2: DNA extraction and DNA measurement

Material: distilled water in erlenmeyer (4°C)

1, 2, 5, and 10 ml pipettes

automatic pipette P-5000

centrifuge (4°C)

water bath (37-38°C)

parafilm

tubes 15 X 150 mm (twice the number of samples to be analysed)

1st series: acido-solubles

2nd series: RNA

tubes 15 X 100 mm for final supernatant fraction

conical tubes 15 ml in ice bath

potter (10 or 15 ml)

becher with ice for potter

becher for discarding

Pasteur pipettes sealed at the extremity

diphenylamine

acetic acid glacial CH3COOH

sulphuric acid H2SO4

Homogenate preparation:

-weigh about 0.08 g of cortex and put in 2 ml of cold distilled water

-homogenize with small potter in ice

-put in tubes identified "DNA homogenates"

-add DNA dilution 1/50 solution in each tubes

Solution preparation:

-$HClO_4$ solutions (these volumes correspond to the quantities required for 24 samples):

$HClO_4$ solution concentration	Required quantity of $HClO_4$ solution	Volume to be made up with distilled water
1.2 M	5.22 ml $HClO_4$ 60%	40 ml
1.0 M	21.76 ml $HClO_4$ 60%	200 ml
0.5 M	*15.00 ml $HClO_4$ 1.0 M	30 ml
0.3 M	*33.00 ml $HClO_4$ 1.0 M	110 ml
0.2 M	*60.00 ml $HClO_4$ 1.0 M	300 ml

*use $HClO_4$ 1.0 M to prepare these solutions.

-KOH solution:

KOH solution concentration	Required quantity of KOH	Volume to be made up with distilled water
0.3 M	18.0 ml KOH 1.0 M	60 ml

-diphenylamine reagent:

Quantity (ml)	Diphenylamine	CH_3COOH (ml)	H_2SO_4 (ml)	Acetaldehyde (ml)
75	1.125	75	1.125	0.375
150	2.250	150	2.250	0.750
200	3.000	200	3.000	1.000
250	3.750	250	3.750	1.250
300	4.500	300	4.500	1.500

-acetaldehyde solution:

16 mg/ml - 100 ml = 1.6 g = 2.04 ml

acetaldehyde density = 0.7834 g/ml

2.04 ml acetaldehyde/ 100 ml solution

DNA extraction:

-2 ml cortex homogenate 1/50 + 4 ml $HClO_4$ 0.3 M for 10 min at 0°C. Mix well with sealed Pasteur pipette

-centrifuge at 2500 rpm for 10 min at 0°C. This give the acido-soluble supernatant fraction

-wash with 3 ml of $HClO_4$ 0.2 M at 0°C. Centrifuge at 2500 rpm for 10 min at 0°C. Take the supernatant fraction and put in tubes identified acido-soluble. Repeat this operation one more time.

-add 2 ml of KOH 0.3 M, mix well and put at 37°C for 1h (shake often). Cool in ice.

-add 1.2 ml $HClO_4$ 1.2 M and let stand for 10 min at 0°C

-centrifuge at 2500 rpm for 10 min at 0°C. This give the RNA supernatant fraction.

-wash with 3 ml of $HClO_4$ 0.2 M at 0°C. Centrifuge at 2500 rpm for 10 min at 0°C. Take the supernatant and put in tubes identified RNA. Repeat this operation one more time.

-drain the tubes

-add 2 ml HClO4 1.0 M, mix well and put at 65°C for 15 min (shake 3 times)

-add 2 ml of distilled water with automatic pipette. Centrifuge at 2500 rpm for 10 min at 0°C.

-take the supernatant fraction and put in tubes identified DNA

-take 0.7 ml of DNA supernatant and put in scintillation vials

-add 10 ml Ecolite

-measure

DNA measurement:

-700 µl DNA supernatant + 300 µl HClO4 0.5 M (prepare two tubes of each)

-under the fume hood, add 2 ml diphenylamine reagent

-shake well

-cover the tubes with glass marble

-incubate at 30°C for 16-20 h

-read at 600 nm with microplate reader

DNA standard:

-stock solution (0.2 ml/mg DNA in 5 mM NaOH)

-take 5 ml stock solution and put 5 ml $HClO_4$ 1.0 M

-under the fume hood, add 2 ml diphenylamine reagent

-shake well

-put in a 70°C water bath for 15 min

-prepare the DNA standard (prepare two tubes of each):

	DNA standard (µl)	$HClO_4$ 0.5 M (µl)
B1		1000
10	100	900
20	200	800
30	300	700
40	400	600
50	500	500

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