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Dietary Composition Alters Gentamicin-Induced Nephrotoxicity in Rats

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ABSTRACT

Gentamicin is an antibiotic of the aminoglycoside family used against aerobic gram positive and negative organisms. Previous studies have shown temporal rhythms in gentamicin-induced renal toxicity characterized by a peak when administered during the resting period and a trough during the active period. Macronutrient composition of food was shown to have a potent impact in modulating circadian rhythms of gentamicin toxicity. In the present study, adult female Sprague-Dawley rats fully adapted to isocaloric 20 % casein-containing, 20% soy-containing (both semi-purified with 10% safflower oil and 58.55% carbohydrate) or a standard chow diet (non-purified with 18.1% mixed proteins, 4.5% fat and 57.3% carbohydrate) were chronically treated for 10 days with a nephrotoxic dose of gentamicin sulfate (40 mg/kg/day, i.p.) or a saline solution given in the middle of their resting period or in the middle of their activity period. Body weights, 24-h, 12-h light and 12-h dark food intakes were measured before (Days 1 to 5) and during treatment (Days 6 to 15). Gentamicin nephrotoxicity indices including serum creatinine, creatinine clearance, urinary proteins, urinary enzymes activities, corticocellular regeneration and cortical accumulation of gentamicin were measured at specific time points during the experiment. Only body weights of rats injected at 1200 h decreased over the last 6 days of gentamicin treatment. Among rats injected with gentamicin during their resting period and conditioned to the casein- and soy-containing diets, significantly higher corticocellular regeneration, serum creatinine and blood urea nitrogen were found compared to rats fed standard chow diet. Total 24-h, 12-h light and 12-h dark food intakes were decreased in gentamicin-treated rats during both resting or activity periods. During the second half of the treatment (Days 12 to 15), a significantly lower 12-h light food intake was found in rats fed the semi-purified casein and soy diets compared to animals fed the standard chow diet. The present study demonstrates that chronic gentamicin-induced renal toxicity varies temporally according to the time of administration, and that a mixed protein diet containing a lower fat level can protects against gentamicin-induced nephrotoxicity.

RÉSUMÉ

Des études antérieures ont démontré que les rythmes temporels de la néphrotoxicité induite par la gentamicine sont caractérisés par un pic lorsque administrée au milieu de la période de repos et une vallée au milieu de la période d'activité. La teneur des diètes en macronutriment s'avère avoir un impact sur la modulation des rythmes circadiens de la toxicité induite par la gentamicine. Dans cette étude, des rates adultes Sprague-Dawley adaptées à des diètes isocaloriques contenant 20% de caséine, 20% de soya (chacune étant semi-purifiée et contenant 10% d'huile de carthame et 58.55% d'hydrates de carbone) ou une diète chow standard (non-purifiée contenant 18.1% de protéines, 4.5% de gras et 57.3% d'hydrates de carbone) ont été chroniquement traitées avec une dose néphrotoxique de gentamicine (40 mg/kg/jour, i.p.) ou avec une solution saline au milieu de leur période de repos (1200 h) ou d'activité (2400 h). Le poids corporel, la prise alimentaire totale, les prises alimentaires de 12-h jour et de 12-h nuit ont été mesurés avant (Jours 1 à 5) et durant le traitement (Jours 6 à 15). Les indices de néphrotoxicité incluant la créatinine sérique, la clairance de la créatinine, les protéines urinaires, l'activité enzymatique urinaire, la régénération corticocellulaire ainsi que l'accumulation corticale de gentamicine ont étés mesurés à des moments précis de l'expérience. Les poids corporels ont diminué durant les 6 derniers jours du traitement chez les rates injectées avec la gentamicine à 1200 h. Une hausse de la régénération cellulaire, de la créatinine sérique et des déchets azotés sériques a été observée chez les animaux injectés avec la gentamicine durant leur période de repos, et conditionnés aux diètes de caséine et de soya, comparativement aux animaux nourris avec la diète chow standard. Les prises alimentaires des 24 heures, de 12-h jour et de 12-h nuit étaient diminuées chez les rates traitées avec la gentamicine au milieu des périodes de repos et d'activité. Au cours de la seconde partie du traitement (Jours 12 à 15), une baisse significative de la prise alimentaire des 12-h du jour a été observée chez les rates nourries avec les diètes semi-purifiées de caséine et de soya par rapport aux animaux nourris avec la diète chow standard. Cette étude démontre que l'induction d'une toxicité rénale par un traitement chronique de gentamicine varie de façon temporelle selon le temps

d'administration, et qu'une diète composée de protéines mixtes à faible teneur en gras peut protéger contre la néphrotoxicité induite par la gentamicine.

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CONTRIBUTIONS OF AUTHORS

The manuscript was written by the first author, Mélanie Paquette, under the guidance of the fifth author, Louise Thibault (Thesis director), the third author, Gaston Labrecque (research collaborator), and the fourth author, Denis Beauchamp (research collaborator). This work was revised by Dr. Louise Thibault, Dr. Gaston Labrecque, and Dr. Denis Beauchamp who used their scientific knowledge in this research area to reinforce the content and the scientific scope of the manuscript. The third, fourth, and last authors provided all the material, supplies, and facilities required to run this experiment. The second author, Isabelle Plante, instructed and helped the first author to perform the cellular regeneration, cortical accumulation of gentamicin, and urinary protein assays. The second author also helped in the preparation of the urine and serum dosages prior to be sent to the biochemical laboratory . 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 authors and by a scholarship from the Fonds de la Recherche en Santé du Québec (FRSQ) held by the first author.

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LIST OF ABBREVIATIONS

AG: Aminoglycosides

h: Hour

i.m.: Intramuscular

i.p.: Intraperitoneal

i.v.: Intravenous

kg: Kilogram

L: Liter

mg: Milligram

min: Minute

mL: Milliliter

NAG: N-acetyl-β-D-glucosaminidase

nm: Nanometer

s.c.: Subcutaneous

βGal: β-galactosidase

γ-GT: γ-glutamyl-transpeptidase

Chapter 1

INTRODUCTION

Aminoglycosides (AG) account for approximately one-fifth of all antimicrobial drugs used in hospitalized patients due to their broad-spectrum activity against aerobic gram positive and negative organisms (Laurent *et al.* 1990). The renal toxicity incidence in 17% of treated patients (Kahlmeter and Dahlager, 1984) motivated the work of several groups of researchers aiming to find effective and safe practices that can alleviate toxicity due to aminoglycosides.

Modification of AG toxicity cannot be obtained by a structural change without compromising its antibacterial action (Ali, 1995). Nowadays, single dosage instead of the traditional multiple dosage regimens of AG is the only clinical approach used to decrease gentamicin toxicity (Beauchamp *et al.*, 1995).

Aminoglycoside-induced renal toxicity is known to display temporal variations in both animals and humans, with a peak observed when the treatment is administered during the resting period and a trough when the treatment is administered during the activity period (Beauchamp *et al.*, 1996; 1997, Lin *et al.*, 1994a; 1996, Prins *et al.*, 1997, Yoshiyama *et al.*, 1992; 1996). Further studies found increased nephrotoxicity in fasted rats compared to those fed *ad libitum* (Beauchamp *et al.*, 1996). In addition, animal subjected to time-restricted feeding periods showed displaced peak and trough of nephrotoxicity following their respective fasting and feeding periods, it was concluded that concurrent food intake with treatment can modulate circadian variations of AG nephrotoxicity (Beauchamp *et al.*,

1997). Recently, Julien *et al.* (2000) reported that chronic gentamicin treatment, administered in the middle of resting and active periods, decreased food intake of rats fed a standard chow diet. Gentamicin was found to be less toxic with lower accumulation into renal cortices of rats fed a casein-rich diet while those fed a lipid-rich diet composed of vegetable shortening and soybean oil exacerbated renal toxicity compared to saline-treated rats (Karzazi *et al.*, 1996). Because macronutrient composition, and especially a protein-rich diet using casein as a single source of dietary protein, showed protective effects against gentamicin-induced nephrotoxicity, it would be interesting to confirm the macronutrient composition that favors renal protection with concurrent gentamicin treatment. Therefore, the present research project aims to investigate the specificity of the protective effect of various dietary protein sources in rats chronically treated with a nephrotoxic dose of gentamicin at times corresponding to the peak (1200 h) and trough (2400 h) of renal toxicity. The hypothesis of this fundamental study is that a casein-containing diet will have a protective effect against nephrotoxicity chronically induced by gentamicin, compared to soy-containing and standard chow diets of similar protein level in rats.

The hypothesis was tested by administering a daily intraperitoneal (i.p.) dose of gentamicin 40 mg/kg, or saline solution (NaCl 0.9%) at 1200 or 2400 h for 10 days to adult female Sprague-Dawley rats adapted to either casein-, soy-containing or standard chow diets.

The first objective of this study was to determine the effect of a chronic treatment with a nephrotoxic dose of gentamicin on body weight, serum albumin and on food intake

over 24-h, 12-h light and 12-h dark periods in each group of rats. The second objective was to assess which diet among casein-, soy-containing and standard chow diets, was protective against nephrotoxicity induced by gentamicin by measuring creatinine clearance, urinary N-acetyl-β-D-glucosaminidase (NAG) and β-galactosidase (βGal), blood urea nitrogen, serum creatinine, the intracortical accumulation of gentamicin, and the incorporation of [^{3}H]-Thymidine in the DNA of renal cortex. The third objective was to test if gentamicin temporal variations in nephrotoxicity induced higher toxicity when rats were injected in the middle of their resting period at 1200 h compared to rats injected in the middle of their activity period at 2400 h, thus supporting previous observations.

Chapter 2

LITERATURE REVIEW

1.0 AMINOGLYCOSIDES

1.1 Pharmacology of aminoglycosides

Aminoglycosides, also called aminosugar-containing antibiotics, have a broad-spectrum activity against infections caused by aerobic gram positive and negative organisms, especially those caused by *Enterobacter, Escherichia, Klebsiella, Proteus* and *Pseudomonas*. This drug family may be considered for the treatment of bacteremia, respiratory and urinary tract infection, infected wounds (surgical and traumatic), bone and soft tissues infections including peritonitis and burns complicated by sepsis (CPS, 1997). Gentamicin (derived from *Micomonospora purpurea* and *Micomonospora echinospora*) and tobramycin (derived from *Streptomyces tenebrarius*) are both AG naturally occurring compounds, whereas netilmicin and amikacin are semi-synthetic derivatives. The semi-synthetic derivatives demonstrate a greater resistance to many bacterial enzymes that usually inactivate gentamicin and tobramycin action (Laurent *et al.*, 1990).

Aminoglycosides invade the outer envelope and the cytoplasmic membrane of bacteria by an oxygen-requiring and energy-dependent process thence, anaerobic organisms are resistant to such antibiotics. They genetically act upon bacteria by an irreversible binding on the 30S ribosomal subunit altering the 30S initiation complex formation inducing conformational change of the ribosome and affecting normal binding sites and slowing down or completely inhibiting growth rate of bacteria. In addition, AG can induce a breakdown of

the 70S initiation complex that is known to be responsible for misreading or/and miscoding during protein synthesis (Tanaka, 1975). The chemical moiety responsible for misreading is the streptamine nucleus, which is an intrinsic part of the structure of AG (Jacoby and Gorini, 1967). Overall, AG affect the three phases of the protein synthesis process: they affect the peptide chain initiation by blocking the 30S initiation complex formation and by inhibiting the ribosomal dissociation (non-reversible binding) and they induce misreading of the genetic code of the bacteria during the elongation and the termination phases of protein synthesis (Jacoby and Gorini, 1967).

1.2 Pharmacokinetics of aminoglycosides

The aminoglycoside family of antibiotics display similar pharmacokinetic parameters. As other drugs primarily eliminated by renal function, gentamicin is physiologically regulated through filtration, secretion and reabsorption and demonstrates a relatively small volume of distribution of approximately 0.22±0.05 l/Kg in humans (Yoshiyama *et al.*, 1996) and 0.28±0.01 l/Kg in rats (Lin *et al.*, 1994 b) that allows the use of one-compartment model to evaluate pharmacokinetic parameters (Table 1). Parenteral routes of administration such as intravenous (i.v), intramuscular (i.m.), intraperitoneal (i.p.), and few others maximize bioavailability of gentamicin. Peak serum concentrations are reached 30 to 90 minutes following an i.m. injection and within 30 minutes after an i.v. injection (CPS, 1997). Gentamicin half-life[1] (t ½) is 2 to 3 h for humans (CPS, 1997; Yoshiyama *et al.*, 1996) and 0.5 to 1.3 h for rats (Lin *et al.*, 1994) in normal conditions, and increases

proportionally as renal function declines. In man, about 25 to 30% of the administered dose of gentamicin is bound by serum protein (CPS, 1997).

The primary objective for dosing regimen using antibiotics such as gentamicin is to maintain drug serum concentrations within a therapeutic interval, which is defined by a serum concentration of antibiotic that is above the minimal effective concentration and below the minimum toxic dosage. However, a large time interval between injections (24 h) contributes to maintaining serum concentration of gentamicin at subtherapeutic concentrations for prolonged periods, which might increase the likelihood of persistent infection (Brater and Chennavasin, 1984). According to a pharmacokinetic convention, the time during which the serum concentration is within the therapeutic interval is a function not only of half-life but also of dose. Doses prescribed in adult patients, without evidence of impaired renal function, are 160 mg once a day or 80 mg twice daily for 7-10 days. The recommendation for an adult weighing less than 60 kg is a single daily dose of 3.0 mg/kg of body weight (CPS, 1997). The drug regimen must also be adjusted according to patients drug serum levels and renal function assessments (Appel, 1990). Different drug regimens are also prescribed for neonates and young patients. Increasing doses will obviously shift the peak and trough of serum concentration of gentamicin, consequently the time spent above the minimal therapeutic interval and the time above the minimum toxic dosage will be increased.

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[1] Time required to eliminate half of the drug plasmatic concentration.

Table 1: Comparison of aminoglycosides' pharmacokinetic parameters in human and rats

Time of administration	t ½ (h)	$AUC_{0-\infty}$ (μg/h/ml)	CL (ml/min)	Vd (L/kg)
Healthy humans				
0800 h	0.88 ± 0.13	96.0 ± 26.2	74.33 ± 21.33	0.22 ± 0.05
2000 h	0.92 ± 0.13	101.4 ± 25.7	69.50 ± 18.17	0.23 ± 0.06
Healthy rats				
1400 h	0.87 ± 0.22	134.0 ± 18.0	1.59 ± 0.22	134.0 ± 18.0
0200 h	0.70 ± 0.07	101.0 ± 12.0	2.10 ± 0.22	101.0 ± 12.0

(Adapted from Lin *et al.*, 1994 b; Yoshiyama *et al.*, 1996)

1.3 Gentamicin-induced nephrotoxicity

1.3.1 Clinical manifestations of nephrotoxicity

Gentamicin's bactericidal action against organisms potentially resistant to beta-lactam[2] antibiotics is followed by prolonged postantibiotic effects (Craig and Vogelman, 1987) including organ-specific toxicity to both cochlear (ototoxicity) and renal proximal tubule cells in addition to serious vestibular side effects. The clinical manifestations of nephrotoxicity consist primarily in the presence of brush-border (γ-glutamyl-transpeptidase (γ-GT)) and lysosomal enzymes (β-galactosidase (βGal) and N-acetyl-β-D-glucosaminidase (NAG)) in the urine followed by polyuria and urine hypoosmolarity or decreased urine concentration capacity (Tilkian *et al.*, 1995) and possibly resulting from AG interference with vasopressin activity (Appel, 1990). Concurrent increase in both blood urea nitrogen (BUN) and serum creatinine is associated with a decrease in glomerular filtration rate (GFR) reflected by a lower creatinine clearance are known to be late manifestations of

[2] A class of antibiotics including penicillin and cephalosporins due to similar structures and pharmacology.

nephrotoxicity. Moreover, notable alterations in proximal tubular cell transport are responsible for glycosuria, proteinuria, and for urinary sodium, potassium, and magnesium concentrations above the normal values (Cojocel *et al.*, 1984).

1.3.2 Development and pathology of nephrotoxicity

Gentamicin is poorly absorbed in the gastrointestinal tract but is actively reabsorbed by pinocytosis and stored in the lysosomes of proximal tubular cells following i.p., i.m. or i.v. routes of administration (De Broe *et al.*, 1984). Gentamicin is distributed extracellularly and can cross the placental barrier whereas it minimally crosses the blood-brain barrier. Gentamicin uptake is done through a multistep process including: binding to the brush-border membrane followed by endocytic uptake and transfer to lysosomes (Beauchamp *et al.*, 1991; Hori *et al.*, 1992). Aminoglycosides are basic compounds and demonstrate a characteristic polycationic structure expected to interact electrostatically with a panoply of cellular acidic and/or anionic components such as phospholipids.

It is known that gentamicin concentration in renal proximal tubules is not correlated with nephrotoxicity but it is generally recognized that renal tubular necrosis is dose-dependent (Bennett, 1989). Gentamicin is eliminated primarily through glomerular filtration and it's concentration appears unchanged in urine, but a small perceptible fraction (3 to 5%) of the injected dose is actively reabsorbed by proximal tubules cells of the renal cortex (Laurent *et al.*, 1990) causing necrosis in the convoluted (S1-S2) portion of renal proximal tubules (Houghton *et al.*, 1976) accompanied by additional uptake across the basolateral

membrane (Pasturiza-Munoze *et al.*, 1979). Drug accumulation into lysosomes can be demonstrated by autoradiography (Silverblatt and Kuehn, 1979) or by Immunogold labeling (Beauchamp *et al.*, 1991) and may reach concentrations that are several-fold higher than the normal serum levels (Giuliano *et al.*, 1984).

In both animals and humans, lysosomal phospholipidosis causes cellular necrosis and alterations in the epithelial cells of proximal tubules and, to a lesser extent, in epithelial cells of distal tubules and collecting ducts. Cojocel *et al.*, (1984) suggested that gentamicin treatment in rats (30 mg/kg/12 h, i.p.) over 7, 14, and 21 days induces ultrastructural changes at the glomerular and/or the tubular level of the kidneys impairing endocytic reabsorption of proteins and their subsequent accumulation. The use of scanning electron microscopy produced evidence that gentamicin induces alterations of endothelial cells by decreasing their density, shape and size of glomerular endothelial fenestrae. Cojocel *et al.*, (1984) reported that gentamicin decreased GFR and impaired renal absorption, leading to an accumulation of low molecular weight proteins along with an increased urinary excretion of sodium and potassium. In normal conditions, low molecular weight proteins are filtered by the glomerulus and almost totally reabsorbed.

1.3.3 Lysosomal alterations

A fairly acidic environment favors electrostatic interactions between AG and the negatively charged phospholipids. Aminoglycosides readily bind to the negatively charged lysosomal phospholipids bilayer since they are exposed to a more acidic pH (~5.4) milieu

then in any other organelles. The presence of the negatively charged phospholipids stimulates phosphatidylcholine degradation mediated by acid sphingomyelinase, phospholipase A1, phospholipase A2 and lipophospholipase (Laurent *et al.*, 1990). However, it is not known whether the catabolism of other polar lipids can be similarly impaired. Phospholipid turnover rate decreases in proximal tubular cells grown in presence of gentamicin, due to the impairment of phospholipid catabolism (Laurent *et al.*, 1990). Aminoglycosides' inhibitory activity on phospholipases causing lysosomal phospholipidosis is characterized by the presence of myeloid bodies (Laurent *et al.*, 1982). In laboratory animals and in humans, an increase in phospholipids content in the renal cortex (Giuliano *et al.*, 1984; DeBroe *et al.* 1984) and an increase in phospholipiduria (Ibrahim *et al.*, 1989) were associated with lysosomal alterations (Figure B, in Appendix). Phospholipids accumulation in lysosomes alters cells stability, leading to organelle rupture and release of harmful components including the drug itself. Evaluating lysosomal phospholipidosis, which precedes overt epithelial damage, can be used to assess AG accumulation in proximal tubule cells by tubular necrosis. Laboratory values such as serum creatinine and BUN reflect GFR and nitrogen balance. The nephrotoxic process is generally reversible upon discontinuation of the drug treatment because of the regenerative capabilities of renal epithelium. Glomerular filtration rate values will appear abnormal only when a large part (>30%) of proximal tubules are necrotic (Kourilsky *et al.*, 1982). It is particularly important to mention that kidney dysfunction occurs when tissue regeneration cannot counterbalance tissue damage induced by a threatening agent.

Phospholipidosis induces an array of tubular alterations such as: 1) the release of brush border and lysosomal enzymes, 2) mitochondrial alterations, 3) tubular necrosis, and 4) the regeneration of tubular functions. Giuliano *et al.*, (1984) characterized morphologically and biochemically the recovery of cortical phospholipidosis and necrosis in renal cells after a loading dose of gentamicin to instantaneously achieve the desired serum level followed by a 12-h continuous drug infusion (infusion/withdrawal pump, series 2200) of gentamicin solution into the jugular vein at 10, 60 and 140 mg/kg at an infusion rate of 19.1 μl/min in adult female Wistar rats. With the 10 mg/kg dose, myeloid bodies were seen inside lysosomes of proximal tubular cells along with a small decrease of lysosomal sphingomyelinase activity, then, both values became normal as the cortical drug level declined. However, contrarily to the 10 mg/kg dose, a dose of 140 mg/kg of gentamicin resulted in a sustained loss of sphingomyelinase activity, increased phospholipids concentration in the renal cortex and an accumulation of myeloid bodies within lysosomes of proximal tubular cells. With the 140 mg/kg dose of gentamicin, tubular regeneration, declined of drug concentration in the renal cortex and decrease in phospholipidosis were respectively observed 24-h (one day), 48-h (two days) and 72-h to 96-h (three to four days) after the end of drug infusion. In general, full recovery may take several weeks (Matthew, 1992) since renal tissue half-life for aminoglycosides is several hundred hours implying a small urinary excretion for weeks following the treatment even after undetectable serum levels (Appel, 1990).

1.3.4 Gentamicin toxic metabolites

Acute AG toxicity on renal cells *in vivo* is well documented whereas no acute toxicity has been reported *in vitro* (Huang *et al.*, 1990). The discrepancy between *in vivo* and *in vitro* results may suggest that toxic metabolite formation as a result of oxidative mechanisms is required for these antibiotics to display toxic actions. On the other hand, Huang *et al.*, (1990) incubated gentamicin along with hepatic enzymes and observed a decrease in cell viability, whereas the antibiotic showed no cytotoxic effects in absence of enzymes. The appearance of nephrotoxicity was delayed for days, even weeks, despite the early peak of the drug in renal tissues. Delays in toxicity onset along with the poor correlation between AG concentration and nephrotoxicity could be explained by the period of time required for induction of the metabolizing enzymes or by the period of incubation required to reach toxic levels. The fundamental role of liver in the formation of toxic metabolites from drugs and xenobiotics is well documented and can be divided into two main types of in vivo reactions. One which is usually associated with microsomal cytochrome P450s, monoamine oxydase and monoxygenase aiming to increase metabolites polarity in order to unable its renal excretion (Crann *et al.*, 1992). Sanders *et al.*, (1993) pre-treated male Sprague-Dawley rats of 6 weeks of age with an inhibitor (P450-dependent) of the drug metabolism prior to a single daily injection of gentamicin (100 mg/kg, s.c.) for 7 consecutive days and found that inhibition of cytochrome P450 in toxic gentamicin metabolites formation did not alleviate gentamicin-induced nephrotoxicity. Consequently,

the researchers suggested that the drug itself and not the cytochrome P450 is responsible for the formation of gentamicin-derived toxic metabolites is responsible to nephrotoxicity.

1.4 Factors affecting gentamicin nephrotoxicity

Beauchamp *et al.*, (1995) and Appel (1990) reported a myriad of risk factors associated with nephrotoxicity in human and laboratory animals, such as advanced age, female gender, hypotension, shock, prolonged or repeated treatment, high creatinine clearance, hypoalbuminemia, impaired hepatic and/or renal functions, and presence of a pyelonephritis, endocarditis, osteomyelitis or gram negative septicemia. Over and above all these risk factors, some can be clinically monitored such as the treatment duration (acute or chronic), the total dose per 24 h, the time of administration, the frequency of administration, concurrent nephrotoxic drug administration and a therapeutic diet along with control for dehydration and hypokalemia.

2.0 BIOLOGICAL RHYTHMS

Concepts of intraindividual temporal variations and biological clock were not considered in ancient biology and medicine practice. A common belief was that any disturbance in the static biologic parameters over time threatened body homeostasis, thus survival. In 1957, Reinberg and Ghata suggested that biological rhythms should be scientifically recognized as intrinsic and fundamental characteristics of living organisms from molecules to individuals. Measurements and dosages taken from organisms were shown to display their own rhythmicity, thus being somewhat predictable. Biological rhythms (Table 2) can be defined as statistically validated physiologic changes recurring with a reproductive waveform, characterized by a maximum (acrophase) and a minimum (bathyphase), as a function of time (Reinberg and Halberg, 1971), and chronobiology is a research area which aims to understand fluctuations of biological rhythms and the mechanisms and/or factors that initiate or regulate them over time. Consequently, the concept of biological "constant" has to be substituted for biological "variable". Biological rhythms are genetically inherited traits, but they are controlled by environmental time cues called synchronizers or zeitbergers. The alternance light/dark over a 24-h period is responsible for the persistence of the most frequently clinically encountered rhythm; the circadian rhythm.

Table 2: Classification of Biological Rhythms

Rhythms	Interval (τ) between peaks	Examples
Ultradian	30 minutes to 20 h	▪ Food intake ▪ Hormone secretion ▪ Progression of sleep stages during night ▪ Response to drugs
Circadian	Months to years	▪ Blood constituents (i.e.: morning rise of blood pressure) ▪ Urinary variables ▪ Sleep-wakefulness
Infradian	~ 24 h	▪ Menstrual cycle ▪ Annual onset of hay fever

(Adapted from Beauchamp and Labrecque, 1999; Reinberg and Halberg, 1971)

2.1 Chronopharmacology

In the early 70's, chronopharmacology emerged from the application of chronobiology in the identification of biologic rhythms in drug effects and kinetics. The main purpose of chronopharmacology is to study the rhythmicity of drug's effects as a function of biological rhythms on parameters that regulate pharmacokinetics and chronoesthetics. Pharmacokinetics parameters includes the maximal drug concentration in plasma (Cmax), the time required to reach Cmax (tmax) in relation to the time of administration (T_0), the half-life (t1/2), the area under the curve (AUC), etc. Chronoesthetics is defined as the response of targeted organs to drug therapeutic qualified as desired effects (chronoefficacy) and undesirable effects (chronotoxicity). The temporal reference of chronopharmacology is

the number of hours after light onset (HALO) for laboratory animals (Reinberg *et al.*, 1991) and usual mean time of wakefulness and sleeping times (7 h and 23 h) for clinical practice in humans. The awareness of existent physiological, toxicological, pharmacokinetics, and pharmacological rhythmicity implies the application of those concepts through chronotherapeutics. The main goals of chronotherapeutics are to optimize drug administration according to peaks of drug efficacy, patient tolerance, and disease's signs and symptoms (Reinberg *et al.*, 1991).

2.2 Renal chronophysiology

The acrophase of diuresis including urinary excretion of protein and electrolytes was consistently observed in all species to occur during the activity period (Cambar *et al.*, 1987). Temporal variations of urinary pH rely on nychthemeral variations of hydrogen ions and ammonium excretion being higher during the resting period which is at night for human and at light for rats. Drug pharmacokinetics will thence be highly influenced by urinary pH with an increased in acidic drug elimination when the urine is alkaline and increased in basic drug elimination when urine is acidic. Consequently, gentamicin excretion is enhanced by low urinary pH (CPS, 1997) during the active period of the subject. In addition, hemodynamic and hormonal factors can regulate renal function as well. Both blood pressure and cardiac output are diminished during sleeping periods leading to a proportional decrease in glomerular filtration reflected by a lower creatinine clearance. A considerable amount of

hormones including cathecolamines, adrenaline and noradrenaline demonstrate circadian variations in regulating renal functions.

2.3 Temporal variations of gentamicin nephrotoxicity

Temporal variations of drug efficacy and nephrotoxicity of AG essentially rely on physiological alterations related to their absorption, distribution and excretion. Drug bioavailability, elimination rate and plasma concentrations are among the factors to be accounted for when predicting toxicity (Table 3). Nakano and Ogawa (1982) were the first to demonstrate temporal variation in gentamicin toxicity when they reported that more male ICR mice died when they were administered a lethal dose of gentamicin (285 mg/kg, s.c.) during their resting period at 14h00 (peak of toxicity) than during their activity period at 02h00 (trough of toxicity). In 1990, Nakano and colleagues reported a circadian rhythmicity of gentamicin kinetics in four weeks old male ICR mice adapted to a 12:12 light:dark cycle (lights on at 0700 h) and injected with a single dose of gentamicin (10 mg/kg, s.c.) at six different time points around 24-h, and in nine healthy men who kept regular diurnal activity and nocturnal rest injected with a single dose of gentamicin (80 mg/day, i.m.) at three different time points of the day. A subsequent experiment also repeated circadian rhythmicity of subchronnic toxicity in male ICR mice (age not mentioned) conditioned as mentioned above but injected with a single dose of gentamicin (220 mg/kg, s.c.) for 18 days at midlight (1300 h) or at middark (0100 h) these results

suggest that the time of administration is critical in gentamicin's chronopharmacokinetics and in the prevention of nephrotoxicity in both animals and humans.

Yoshiyama *et al.*, (1992) showed that chronic administration of gentamicin (60mg/kg, s.c.) for 8 consecutive days in adult male Wistar rats adapted to a 12:12 light:dark cycle (light on at 0700 h) induced greater tubular necrosis and increased urinary enzyme NAG excretion by 50 % from baseline when given in the middle of the resting period (at 1300 h) than in the middle of the activity period (at 0100 h). Similar results were found in adult female Sprague-Dawley rats adapted to a 14:10 light:dark cycle (lights on at 0600 h) where maximal nephrotoxicity was observed when tobramycin (40 mg/kg, i.p.) was injected in the middle of the resting period (1400 h) and minimal nephrotoxicity was observed when injected in the middle of the activity period (0200 h) (Lin *et al.*, 1994a;1996).

Evidence of temporal variations in AG's nephrotoxicity in humans is scarce. A prospective study was conducted by Prins et al., (1997) using 221 severely infected patients treated with a single dose of gentamicin or tobramycin (4 mg/kg, i.v.). Patients were separated into three groups according to the first time of drug administration: one group injected during the resting period (0000 to 0730 h), one group injected during the early activity period (0800 to 1530 h), and the other group injected during the late activity period (1600 to 2330 h). Although, peaks and troughs of drug serum concentrations were similar among groups, nephrotoxicity reflected by an increased serum creatinine, occurred significantly more often when AG were injected during the resting period (0000 to 0730 h).

Table 3: Effects of Temporal Variations on Gentamicin Toxicity

Parameters of toxicity	Time of maximal value	References
Laboratory animals		
• Mortality rate	1300 h	Nakano and Ogawa, 1982
	1400 h	Pariat *et al.*, 1984
	1300 h	Nakano *et al.*, 1990
• Urine output	1400 h	Beauchamp *et al.*, 1996
• Urinary enzyme excretion		
-NAG	1400 h	Pariat *et al.*, 1988
	1400 h	Beauchamp *et al.*, 1996
-βGal	1400 h	Pariat *et al.*, 1988
	1300 h	Yoshiyama *et al.*, 1992
-γ-GT	1400 h	Beauchamp *et al.*, 1996
	1400 h	Beauchamp *et al.*, 1997
• Cellular regeneration of renal cortex	1400 h	Beauchamp *et al.*, 1996
	1300 h	Karzazi *et al.*, 1996a
• Serum creatinine	1300 h	Karzazi *et al.*, 1996a
	1300 h	Julien *et al.*, 2000
• Creatinine clearance	1300 h	Julien *et al.*, 2000
Humans		
• Serum creatinine	1600 h – 2330 h	Prins *et al.*, 1997

(Adapted from Beauchamp and Labrecque, 1999) Julien et al., 1999

The frequency of administration is an important factor that can be clinically monitored to alleviate gentamicin-induced nephrotoxicity. Indeed, Laurent *et al.*, (1983) demonstrated that administrating gentamicin (10 mg/kg, i.p.) to young adult female Sprague-Dawley rats in a single injection (once every 24 hours) decreased AG accumulation

in renal cortices, severity of tubular necrosis and renal functional impairment when compared to equivalent doses fragmented into three injections during the day (every 8 hours). Furthermore, using 260 adult male Fisher 344 rats, Wood *et al.* (1988) compared the influence of the administration of the same total daily s.c. dosage of tobramycin administered at multiple intervals across the circadian cycle: 10 mg/kg every 4 h, 20 mg/kg every 8 h, 30 mg/kg every 12 h, 60 mg/kg every 24 h. They reported that the drug was as effective, but less nephrotoxic, when given once a day rather than four times a day. A similar study design using 40 female mongrel dogs performed by Reiner *et al.*, (1978) concluded that continuous infusion of gentamicin leads to a greater decrease in renal function compared to once daily injection of the same total dose. The relationship between the interval of administration and toxicity can be explained by the postantibiotic effect of AG. Since multiple daily doses are not mandatory for full display of their therapeutic activity, decreasing the number of daily doses allows optimal antimicrobial activity and limits cytotoxic effects.

Circanual variations in AG nephrotoxicity were demonstrated by Dorian *et al.*, (1985) in rats injected rats with amikacin (400 mg/kg) for 7 consecutive days, reporting maximal toxicity during the active period in the fall and during the resting period in the summer.

3.0 DIETARY MODULATION OF GENTAMICIN-INDUCED NEPHROTOCITY

3.1 Feeding and drinking rhythms

The regulation of the awakening and sleeping cycle around the day occurs accordingly through the light and dark alternance. Feeding and drinking behaviors of humans and animals can solely be observed during their respective diurnal and nocturnal activity periods. Consequently, circadian rhythms are displayed in food and water ingestion. For instance, healthy rats usually consume more than 70% of their food and water requirements during their nocturnal activity period (Zucker, 1971) and at least 70 % of their water intake is closely related to meal consumption (Fitzsimons and LeMagnen, 1969). It is scientifically recognized that the presence of food or meals can alter but cannot generate circadian rhythms. Indeed, time of food intake, food restrictions and fasting are all aspects that can be manipulated in order to understand the protective effects of foods against different induced stresses such as gentamicin treatments.

Temporal modulations of nephrotoxicity and rhythmic pattern of food and water ingestions were studied in gentamicin-treated rats Julien *et al.*, (2000). Dietary and water intakes were measured during a 5-day pretreatment and a 5-day treatment period in adult female Sprague-Dawley rats treated with gentamicin (80 mg/kg/day, i.p.) at 1300 or 0100 h, corresponding respectively to the middle of the rest period and the middle of the activity period. Temporal variations of gentamicin-induced nephrotoxicity parameters concur with previous studies (Nakano and Ogawa, 1982; Pariat *et al.*, 1984; Beauchamp *et al.*, 1996; 1997) being significantly higher when rats were injected in the middle of their resting period

compared to rats injected in the middle of their activity period supporting peak and trough of gentamicin-induced nephrotoxicity. Gentamicin administration at both times of injections (1300 and 0100 h) resulted in a decrease in the 24-h food intake. More precisely, gentamicin-injected rats at 0100 h decreased their usual maximal food intake in the late dark period, making the early dark period their alternative maximal food intake period. The authors concluded that gentamicin inhibits and alters temporal distribution of food intake and suggested that this decrease in food intake may exacerbate gentamicin-induced nephrotoxic effect.

3.2 Presence of food and food access schedule

Since minimal gentamicin-induced toxicity occurs during the activity period where food intake is maximal, Beauchamp *et al*, (1996) evaluated the effect of fasting on temporal variations of gentamicin in 28 normally fed with a standard chow diet and 28 fasted (12-h before and 24-h after injection) young adult female Sprague-Dawley rats adapted to a 14:10 light:dark schedule (lights on at 0600 h). Rats were injected with a single i.p injection of saline (NaCl, 0.9%) or gentamicin (150 mg/kg, i.p.) at times corresponding to the peak (1400 h, middle of the resting period) or the trough (0200 h, middle of the activity period) of toxicity. Temporal variations in nephrotoxicity were demonstrated through increased 24-h urinary excretion of βGal, NAG, γ-GT and higher cortical gentamicin accumulation in normally fed rats given an acute treatment at 1400 h than at 0200 h. Antagonistic results were found in fasted rats treated with the same dose of gentamicin at the same time points

of the day where gentamicin induced greater renal toxicity compared to normally fed rats but no temporal variations in gentamicin toxicity were observed. The authors concluded that fasted rats were more susceptible to renal toxicity, therefore suggested that dietary intake during the active period of rats is of crucial importance for the temporal modulation of gentamicin-induced nephrotoxicity. The mechanisms by which fasting increased gentamicin toxicity could be linked to the lower creatinine clearance, higher serum creatinine levels and larger accumulation of gentamicin in renal cortices in the fasting state. Beauchamp *et al.*, (1996) hypothesized also that the presence of food may act as modulator in temporal variations of gentamicin nephrotoxicity by shifting the acrophase (peak) and bathyphase (trough). Unfortunately, this shift could not be further characterized because only two time points of the day were examined in this experiment.

Song *et al.*, (1993) investigated the influence of feeding schedule and time of injection on chronopharmacological parameters of gentamicin in 6 week old ICR mice adapted to a 12:12 light:dark cycle (lights on at 0700 h) and fed *ad libitum* with food and water or on a time restricted schedule (food available 8 hours during the light phase) for 1 or 14 days prior to drug administration. Gentamicin kinetics was studied in mice injected a single 180 mg/kg, s.c. dose and subchronic gentamicin toxicity studies evaluated 180 mg/kg, s.c. for 14 days or 220 mg/kg, s.c. for 18 days. A significant dosing-time dependency was found for mortality rate and body weight with a higher mortality rate and greater body weight loss when gentamicin was administered in the middle of their resting period than in the middle of their activity period. The circadian rhythm of gentamicin toxicity and it's

kinetics coincided well together. Time-restrictively fed mice had a lower clearance and had a higher toxicity in the middle of their activity period than mice fed *ad libitum*. Overall, the authors concluded that manipulating feeding schedule induced perturbations in gentamicin's toxicity rhythm by modifying drug kinetics.

Beauchamp *et al.* (1997) investigated, in young adult female Sprague-Dawley rats adapted to a 14:10 light:dark cycle (lights on at 0600 h), if restricted feeding schedules with food availability from 0800–1600 h, 1600–0000 h, and 0000-0800 h could modify temporal variations of gentamicin nephrotoxicity. Rats were treated for 4 and 10 days with gentamicin (40 mg/kg/day, i.p.) or a saline solution (NaCl, 0.9%) at either 0700, 1500, 1900, or 0100 h according to the aforementioned restricted feeding schedules. Results indicated that time-restricted food access schedule were more potent in displacing peaks and troughs of gentamicin-induced renal toxicity than the light:dark cycle with recurrent minimal gentamicin toxicity when rats are injected during feeding periods and maximal toxicity associated with fasting periods.

3.3 Dietary macronutrients

The presence of food itself along with gentamicin injection was previously shown to modulate gentamicin kinetics and nephrotoxicity and results of restrictive feedings studies correlated this finding. Consequently, new concerns emerged as to whether or not the dietary composition, source, amount and ratio offered to rats can enhance the protective

effect of food against gentamicin-induced nephrotoxicity. Is there an ideal dietary recommendation during such antibiotic treatment?

Karzazi *et al.* (1998) studied adult female Sprague-Dawley rats adapted for 14 days to a 14:10 light:dark cycle (lights on at 0700 h) and fed either a standard rat chow, a protein-rich (45% rat chow and 55% calcium free casein high purified nitrogen) or a lipid-rich diet (60% rat chow, 30% vegetable shortening and 10% soybean oil) prior to receiving an acute injection of gentamicin (150 mg/kg, i.p.). This experimental study evaluated the influence of macronutrient-rich diets on gentamicin-induced nephrotoxicity and found that rats fed the protein-rich diet had a lower excretion of urinary enzymes (NAG and γ-GT) compared to rats fed with the lipid-rich or the standard rat chow diet regimens. In addition, the protein-rich diet induced lower gentamicin accumulation in renal cortices compared to rats fed the lipid-rich diet. The authors also reported higher body weights, lower water intake and lower diuresis in rats fed the lipid-rich diet, which confined higher susceptibility to lipid peroxidation and inducing higher nephrotoxicity. Overall, they concluded that the protein-rich diet, made of 55% casein as the main protein source, could be protective against acute renal toxicity of gentamicin.

3.4 Dietary proteins

There is a challenging possibility that the natural history of renal pathogenecity could be modified by manipulating the quality and the quantity of dietary protein content of diets. The main objective with gentamicin antibiotic treatment is to provide an adequate

balance of dietary protein in order to avoid protein malnutrition and to halt or attenuate the progression of drug-induced nephrotoxicity. A literature review by Klahr *et al.* (1983) of experimental studies in animals and humans clearly depicts that excessive dietary protein intake accelerates progression of renal diseases. High protein intake is known to increase renal blood flow and GFR producing hyperperfusion and hyperfiltration while the mechanisms by which low protein intake attenuates kidney disease are not clear yet. Osborne *et al.* (1926-27) reported that renal hypertrophy but not chronic renal disorder was attributable to protein intake exceeding one-third of the food fuel of the ration presented to healthy rats (strain and gender not mentioned) and this response was reproduced in various dietary proteins including wheat, corn, casein, liver and navy beans. A study by Newburgh and Curtis (1928) reported renal structural damage in healthy young white rats (gender not mentioned) fed casein, beef muscle, beef liver or vegetable containing diets varying from 75 to 80% in protein content. It was found that the degree of renal injury was primarily determined by the type of protein and secondarily by its concentration and the length of the feeding period. Rats fed diets containing 75% of dried liver developed renal lesions in less than 1 year, but the same amount of casein-containing diets fed for 16 months caused only moderate tubular injuries. Diets containing beef muscles as protein source were intermediate in its renal effects. The authors explained that high protein diets might induce renal injury through the extra work required by the kidneys for the removal of unusually high amounts of nitrogenous end-products.

On the other hand, an interesting linkage between glomerular hyperfiltration and structural changes was suggested in studies including dietary protein restriction. In fact, feeding low protein diets to rats with partial kidney ablation prevented the increase in glomerular plasma flow and capillary pressure which lead to hyperfiltration. In addition, accompanying proteinurea and structural changes were less severe. Although more work is necessary in this area, Dworkin *et al.*, 1983 suggested that varying the source of dietary protein may affect vasoconstrictor and/or vasodilatory substances secreted by the kidney. Whiting *et al.* (1988) worked with adult male Sprague-Dawley rats fed *ad libitum* with either a standard Oxoid 18% protein diet or a low 5% protein diet (protein source and adaptation period not mentioned) chronically administered with a single daily dose of gentamicin (120 mg/kg, i.p.) for 10 consecutive days. A lower gentamicin-induced toxicity was found in rats fed the 5% protein diet as shown by a greater creatinine clearance rate, a decrease in NAG activity and less marked histological changes compared to rats receiving the 18% protein diet. In contrast, Grauer *et al.*, (1994) adapted Beagle dogs for 21 days to diets containing either 9.4%, 13.7% or 27.3% protein (protein source not mentioned). Following this adaptation period, dogs fed the 27.3% protein diet had a higher creatinine clearance and urinary excretion of protein compared to dogs fed the 9.4% protein diet. Thereafter, dogs were injected with gentamicin (10 mg/kg, i.m.) every 8 hours for 8 consecutive days. Dogs fed the 27.3% protein diet had higher creatinine clearance, lower serum creatinine concentration, lower clearance of sodium and lower urinary excretion of NAG compared to dogs fed the 13.7% and the 9.4% protein diets. In addition, dogs fed the

27.3% protein diet had a lower excretion of urinary potassium and protein than dogs fed the 9.4% protein diet. Overall, proximal tubular necrosis was more severe in dogs fed the 13.7% compared to those fed the 27.3% protein diet, however there were no difference in cortical concentration of gentamicin among the three dietary groups.

Huang and Fwu (1992) investigated the effect of various dietary protein levels on tissue lipid peroxidation and antioxidative enzyme activities in Long-Evans male weanling rats adapted to a 12:12 light:dark cycle and fed *ad libitum* with 6, 8, 12, or 20% lactalbumin diets for 6 weeks. The results showed a proportional reduction in antioxidative enzyme activities and a consequently enhanced tissue lipid peroxidation with the dietary lactalbumin content. Moreover, they also found that rats fed diets containing the largest concentration of lactalbumin (20%) had increased antioxidative enzyme activities (catalases, glutathione peroxidases (GSHPx) and superoxide free radicals (SOD)) in both erythrocytes and hepatocytes compared to rats fed with the other 6, 8 and 12% protein diets allowing a decrease in tissue lipid peroxidation by maintaining the balance between the antioxidative defense system and oxidative stress. In other words, elevated degree of protein malnutrition resulting from the lower protein diets generally correlated with the extent antioxidative defense system impairment making cells more prone to oxidative destruction, suggesting that insufficient dietary protein consumption might result in enhanced tissue lipid peroxidation. The effect of various dietary protein sources on hepatic enzyme activities was evaluated by Iritani *et al.*, (1986) in experiments conducted with 5 week old male Wistar rats adapted to a 12:12 light:dark cycle (lights on at 0700 h) fed fat-free diets containing

vegetable sources of protein such as soybeans or gluten and animal sources of protein such as casein or fish. Rats fed the vegetable proteins, limited in amino acids, showed decreased lipogenic enzyme activities (glucose-6-phosphate deshydrogenase, malic enzyme, acetyl-CoA carboxylase and fatty acid synthetase) compared with rats fed diets containing complete sources of protein such as casein or fish.

Andrews and Bates (1987-88) examined the effect of dietary protein on renal function, both prior and during a 6 days gentamicin treatment (150 mg/kg, s.c.) in adult male Sprague-Dawley rats fed a Ralston Purina Company purified test isocaloric diets (casein being the protein source) with either low-protein (5%), normal-protein (20%) or high-protein (60%) contents. Rats were conditioned for 10 days to their respective dietary regimens, immediately after the first gentamicin injection some rats conditioned to the 20% protein diet were switched to either the 5 or the 60% protein diets, and some of the rats conditioned to the 60% protein diet were switched to the 5% protein diet. The results showed that dietary protein content of the diet both prior and following gentamicin administration can affect renal function, histology and survival. Uptake of gentamicin in renal cortices decreased as dietary protein percentage in the diet increased. However, rats fed the 60% protein diet during the entire experiment exhibited no significant improvement compared to rats fed the 20% protein diet. Interestingly, rats conditioned to a 60% dietary protein diet and switched to a 5% dietary protein diet demonstrated improvements in both mortality rate and renal function while rats switched from normal to high dietary protein showed a drastic increased in mortality rate.

Since the above studies were conducted in different animal species, using different percentages of protein and different sources of protein, the protective effect against AG nephrotoxicity can be generalized to protein. A clear relationship exists between phospholipidosis and nephrotoxicity but it is still unclear why and how different sources of protein may act to alleviate nephrotoxicity.

3.5 Suggested mechanisms for the protective effect of proteins against gentamicin-induced nephrotoxicity

There is evidence that hepatic formation of antioxidants such as glutathione peroxidase (GSH) is related to nutritional conditions, essentially cysteine (a sulfur-containing amino acid) content of the diet (Wendel *et al.*, 1990; Taylor *et al.*, 1996). Furthermore, Tateishi (1990) suggested a quantitative relationship between cysteine content of the diet and increase of GSH in liver and showed that GSH increases in the liver proportionally to the amount of cysteine supplemented for 12 hours in rats fed a protein-free diet for 40 hours. In 1997, Hunter and Grimble examined the mechanisms explaining the fall and replenishment of hepatic GSH concentrations in rats fed insufficient or adequate amounts of sulfur amino acids in their diets. Young male Wistar rats were fed *ad libitum* with diets containing either 200 g of casein and 8 g of L-cysteine/kg (normal protein diet) or 80 g of casein supplemented with 8 g of L-cysteine/kg or isonitrogenous amounts of L-methionine or L-alanine (low protein diets) for 8 days. Rats fed the low protein diet supplemented with L-alanine displayed the lowest hepatic GSH concentrations and in

contrast to the other diets, this diet did not allow replenishment of hepatic GSH due to its inadequate sulfur amino acid content. In addition, the same study reported a higher increase in GSH concentrations when L-methionine rather than L-cysteine sulfur amino acids was supplementing the low-protein diets.

In rats, some anionic polypeptides such as poly-L-aspartic acid and poly-L-asparagine acid seem to have a protective effect against aminoglycoside-induced nephrotoxicity. In rats, co-administration of poly-L-aspartic acid (500 mg/kg) and gentamicin (100 mg/kg) largely suppressed nephrotoxicity by decreasing aminoglycoside-induced lysosomal phospholipidosis, phospholipiduria and tubular necrosis without decreasing cortical concentrations of gentamicin when compared to gentamicin injected alone (Ramsammy *et al.*, 1989). Poly-L-ascorbic acid can disrupt cortical intracellular processes that usually trigger the injury cascade conducting to cellular necrosis. Similar results were reported by Beauchamp *et al.*, (1986) in adult female Sprague-Dawley rats infused with a mixture of 100 mg/kg gentamicin and 250 mg/kg of either poly-L-aspartic acid or poly-L-asparagine. The main observation was that these amino acids completely prevented diminution of creatinine clearance and attenuated the severity of proximal tubular cell necrosis. For instance, poly amino acids can cancel the inhibition of phosphatidylcholine hydrolysis by lysosomal phospholipases induced by gentamicin. It is important to note that in the study of Beauchamp *et al.*, (1986), the poly amino acids dosages along with gentamicin were administered in a 5:1 ratio, which can only be reached using dietary supplementation.

Chapter 3

MANUSCRIPT

Dietary Composition Alters Gentamicin-Induced Nephrotoxicity in Rats

Dietary Composition Alters Gentamicin-Induced Nephrotoxicity in Rats

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ABSTRACT

PAQUETTE, M., I. PLANTE, G. LABRECQUE, D. BEAUCHAMP AND L. THIBAULT. Dietary composition alters gentamicin-induced nephrotoxicity in rats. PHYSIOL BEHAV XX(X) 000-000, 2000.- Previous studies have shown temporal rhythms in gentamicin-induced renal toxicity characterized by a peak when administered during the resting period and a trough during the active period, can be modulated by macronutrient composition of food. In the present study, adult female Sprague-Dawley rats fully adapted to isocaloric 20% casein-, 20% soy-containing (both semi-purified with 10% safflower oil and 58.55% carbohydrate) or a standard chow diet (non-purified with 18.1% mixed proteins, 4.5% fat and 57.3% carbohydrate) were chronically treated for 10 days with a nephrotoxic dose of gentamicin sulfate (40 mg/kg/day, i.p.) or a saline solution given in the middle of their resting period or in the middle of their activity period. Body weights, 24-h, 12-h light and 12-h dark food intakes were measured before (Days 1 to 5) and during treatment (Days 6 to 15). Gentamicin nephrotoxicity indices including serum creatinine, creatinine clearance, urinary proteins, urinary enzymes activities, corticocellular regeneration and cortical accumulation of gentamicin were measured at specific time points during the experiment. Only body weights of rats injected at 1200 h decreased over the last 6 days of gentamicin treatment. Among rats injected with gentamicin during their resting period and conditioned to the casein- and soy-containing diets, significantly higher corticocellular regeneration, serum creatinine and blood urea nitrogen were found compared to rats fed standard chow diet. Total 24-h, 12-h light and 12-h dark food intakes were decreased in gentamicin-treated rats during both resting or activity periods. During the second half of the treatment (Days 12 to 15), a significantly lower 12-h light food intake was found in rats fed the semi-purified casein and soy diets compared to animals fed the standard chow diet. The present study demonstrates that chronic gentamicin-induced renal toxicity varies temporally

according to the time of administration, and that a mixed protein diet containing a lower fat level can protects against gentamicin-induced nephrotoxicity.

Key words: macronutrients - rhythms – food intake – renal toxicity – temporal variations

1.0 INTRODUCTION

Aminoglycosides (AG) account for approximately one-fifth of all antimicrobial drugs used in hospitalized patients due to their broad-spectrum activity against aerobic gram positive and negative organisms (Laurent *et al.* 1990). The renal toxicity incidence in 17% of treated patients (Kahlmeter and Dahlager, 1984) motivated the work of several groups of researchers aiming to find effective and safe practices that can alleviate aminoglycosides' toxicity.

Modification of AG toxicity cannot be obtained by a structural change without compromising its antibacterial action (Ali, 1995). Nowadays, single dosage instead of the traditional multiple dosage of AG is the only clinical approach used to decrease gentamicin toxicity (Beauchamp *et al.*, 1995).

Aminoglycoside-induced renal toxicity is known to display temporal variations in both animals and humans, with a peak observed when the treatment is administered during the resting period and a trough when the treatment is administered during the activity period (Beauchamp *et al.*, 1996; 1997, Lin *et al.*, 1994a; 1996, Prins *et al.*, 1997, Yoshiyama *et al.*, 1992; 1996). Further studies conducted in our laboratories using fasting and restricted food access schedule showed that concurrent food intake with treatment can modulate circadian variations of AG nephrotoxicity (Beauchamp *et al.*, 1996; 1997). Those observations raised new concerns as to whether or not the diet composition offered to rats could enhance the protective effect of food against gentamicin-induced nephrotoxicity. A subsequent study was conducted in adult female Sprague-Dawley rats

conditioned to macronutrient-rich diets and given an acute dose of gentamicin (150 mg/kg, i.p.). Gentamicin levels were found to be lower in the renal cortex of rats fed a standard chow diet added with 55% casein compared to rats fed either a standard chow diet added with a 30% vegetable shortening and a 10% soybean oil mixture or a standard chow diet (Karzazi *et al.*, 1996). More recently, we reported that chronic gentamicin treatment, administered at a dose of 80 mg/kg i.p. for 5 consecutive days to adult female Sprague-Dawley rats, decreased 24-h food intake of standard chow diet (Julien *et al.*, 2000). In addition, the study demonstrated that chronic gentamicin treatment could disrupt the bimodal distribution of the nychthemeral feeding patterns during the treatment period by decreasing maximal food intake previously observed at the end of the activity period, making the beginning rather than the end of the activity period the maximal food intake period.

Although the protective effect of food, through its availability and its macronutrient composition, against AG nephrotoxicity is not fully understood, it has been tested using casein as a single source of dietary protein. Therefore, the present research project aims to expand previous findings on circadian variations and dietary influence on gentamicin nephrotoxicity parameters (including gentamicin cortical levels, cellular regeneration, creatinine clearance, serum creatinine, blood urea nitrogen (BUN), diuresis, urinary enzymes and urinary proteins) by investigating the specificity of the protective effect of the dietary protein source. Adult female Sprague-Dawley rats adapted to semi-purified diets containing either 20% casein or 20% soy as the protein source or a standard chow diet were

chronically treated for 10 consecutive days with a nephrotoxic dose of gentamicin (40 mg/kg, i.p.) at times corresponding to the peak (1200 h) and trough (2400 h) of renal toxicity.

2.0 MATERIALS AND METHODS

2.1 Animals and diets

Ninety-six adult female Sprague-Dawley rats (Charles River Breeding Laboratories, St-Constant, Quebec, Canada) with initial body weights varying between 225 and 250g were used. Animals were housed in single standard wired-bottom cages, in a room with controlled temperature (22°C) and humidity (68%) and equipped with an automatic dimmer set for a 12-h light/12-h dark cycle schedule with lights on at 0600 h. This light/dark cycle was chosen to allow comparison with most of the recent literature. The rodents were provided *ad libitum* with tap water and a complete semi-purified granulated diet with 20% casein, or 20% soy as protein sources (Table 1) or commercial non-purified standard chow (rodent laboratory chow 5075, Charles River Breeding Laboratories, St-Constant, Quebec, Canada). The standard rat chow, composed of 18.1% crude proteins, 4.5% crude fat, 3.5% crude fiber, 6.7% ash and 2.5% added minerals, was purchased in pellets and granulated in our laboratory by a Deluxe Moulinex Grinder model 133. Granular diets were prepared fresh on alternate days by adding a fixed amount of water to the 3 powdered diets, stirring to obtain a granular consistency and leaving to dry overnight for a complete evaporation of the added water (Mok *et al.*, 1999).

2.2 Experimental design

Animals were conditioned to environment, individual housing, light/dark cycle and their respective diets for 14 days prior to the beginning of the experiments. The animals were randomly divided into two groups (n = 48): one group scheduled to be injected in the middle of its resting period at 1200 h and the other group in the middle of its activity period at 2400 h. Injection time was designed to correspond to the peak and trough of renal toxicity induced by gentamicin (Nakano and Ogawa, 1982; Nakano 1990; Yoshiyama *et al.*, 1992; Lin *et al.*, 1994;96). Each group was subdivided into three dietary groups (n = 16): one group fed the casein-containing diet, the second group fed the soy-containing diet and the third group fed the standard diet.

Daily food intake (g) for the 12-h dark (measured at 0600 h) and 12-h light (measured at 1800 h) periods were gathered daily throughout the experimental period (Days 1 to 15) using a Mettler PM 4600 balance. A five-day baseline period (Days 1 to 5) was determined to assess the rhythms of the food intake in 12-h dark and the 12-h light periods under normal conditions. The same food intake measurements were taken over the antibiotics treatment period (Days 6 to 15). Body weights (g) were measured at the end of the dark period (0600 h) every other day during the adaptation period (Days -14 to 0) and every day during the remaining of the experiment (Days 1 to 15). The animals were transferred into metabolism cages on the last day of the adaptation (Day 0) and on the last five days of the treatment period (Days 11 to 15) for the collection of 24-h urine samples.

Rats were chronically treated for 10 consecutive days (Days 6 to 15) with a daily intraperitoneal (i.p) injection of 40mg/kg of body weight of gentamicin sulfate (generously provided by Schering Canada Inc., Pointe-Claire, Quebec, Canada) or an equivalent volume of a saline solution (NaCl 0.9%). Doses were adjusted daily to individual body weight fluctuations of rats from both treated and control groups. Thirty-six to forty-eight hours following the last gentamicin or saline injection, the animals were sacrificed by decapitation. The fact that the group injected at 1200 h was killed 12 hours later than the group injected at 2400 h has no consequence on nephrotoxicity data (Julien *et al.*, 2000). Exactly 1 hour prior to decapitation each rat was injected with [^{3}H]-thymidine (200μCi: Amersham, Oakville, Ontario, Canada) in order to measure cellular regeneration. Trunk 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 performed, and both kidneys were removed and dissected to recuperate the renal cortex. Tissues were placed on dry ice immediately, and kept at -80°C for future analysis. The experiment was conducted in spring of 1999.

2.3 Biochemical analysis

Blood analysis for serum creatinine, blood urea nitrogen (BUN) and albumin were performed in the biochemistry laboratory of the Centre Hospitalier de l'Universite Laval (CHUL) using an automated enzymatic method (Vitros 950) analyzer. Since urinary creatinine is chiefly excreted by glomerular filtration, serum creatinine and 24-hour urine creatinine excretion can be used to estimate glomerular filtration rate. Creatinine clearance

was calculated by multiplying urine creatinine concentration by the 24-h diuresis measurement and by dividing this product by the serum creatinine concentration. Albumin was quantitatively measured by a colorimetric method using bromocresol green (BCG) and the BCG-albumin complex was read at 630 nm wavelength. Quantification of urinary proteins was performed using the Protein Assay Reagent Kit that is a detergent compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein.

2.4 Enzymuria

Rats were placed in individual metabolic cages on the last day of adaptation (Day 0) which were used as the baseline for urine volume and enzymuria activity to be compared with the last day of treatment (Day 15) of 24-h urine collection in mineral oil. All urine samples were immediately centrifuged after being collected, the remnant volume were recorded and enzyme activities for β-galactosidase (βGal) and N-acetyl-β-glucosaminidase (NAG) were determined within two hours following urine collection using Maruhn colorimetric method (1976). This method consists of using those reactants as catalysers by using the absorbency of the degradation product, 4-nitrophenol, with a spectrophotometer at 405 nm wavelength. In other words, 4-nitrophenyl-β-D-galactopyranoside will be catalyzed by βGal and the absorbency of 4-nitrophenol + β-D-galactopyranoside degradation will be measured. Similar reaction occurs for 4-nitrophenyl-N-acetyl-β-glucosaminidase is catalyzed by NAG and the absorbency of 4-nitrophenol + β-D-glucosaminidase degradation was measured.

2.5 Cortical accumulation of gentamicin

The concentration of gentamicin in the renal cortex was determined by a standard microbiological assay, with *Bacillius subtilis* ATCC 6633 used as the test organism (Tardif *et al.*, 1990).

2.6 Corticocellular regeneration

Loss of tubular epithelium due to a nephrotoxic injury is characterized by an increased proliferation of epithelial cells also known as "tubular regeneration". [^{3}H]-Thymidine was intraperitoneally injected to rats exactly one hour preceding the sacrifice since aminoglycosides induce a dose-related increase of corticocellular proliferation. Renal cortices were homogenated in distilled water using a rotating potter before extracting DNA (Laurent *et al.*, 1983). Cellular regeneration was evaluated by measuring the amount of [^{3}H]-Thymidine incorporation into DNA of the renal cortices.

2.7 Statistical analysis

Total 24-h food intakes (g), food intakes (g) for the 12-h light period and for the 12-h dark period, and body weights (g) throughout the baseline and treatment periods (Days 1 to 15) were analyzed separately using a two-way repeated measures ANOVA, separated according to the two time of the day at which the treatment were administrated, with treatment (gentamicin *versus* saline), and diet (casein-containing *versus* soy-containing *versus* standard chow) as the main effects. Significant main effects and interactions food body weight and food intake patterns were analyzed using Tukey's studentized range test for multiple comparisons.

The nephrotoxicity parameters including serum creatinine, creatinine clearance, urinary proteins, urinary enzyme activities, BUN, albumin and cellular regeneration were analyzed by a three-way repeated measures ANOVA with treatment (gentamicin *versus* saline), time of injection (1200 *versus* 2400 h) and diet (casein-containing *versus* soy-containing *versus* standard chow diet) as the main effects. Nephrotoxicity data for cortical accumulation of gentamicin were analyzed by a two-way repeated measures ANOVA with time of injection and diet as the main effects. The interactions between treatment, time of injection and diet were also tested. Significant main effect differences were tested using Scheffe's test for multiple comparisons. Results are presented as means ± standard error of the mean (SEM). A probability of less than 0.05 was considered significant. The programs used for statistical analysis were Super ANOVA (version 1.11, Abacus Concepts Inc., Berkeley, CA) and SAS (version 6.12).

RESULTS

3.1 Nephrotoxicity parameters

3.1.1 Cellular regeneration

A significant interaction between time of injection (1200 or 2400 h) and treatment (gentamicin or saline) was found for cellular regeneration, $F(1,84)=9.01$, $p<0.005$. Gentamicin treatment at 1200 h resulted in significantly higher [^{3}H]-Thymidine incorporation into renal cortical cells compared to the treatment at 2400 h. A significant interaction between treatment and diet (casein-containing, soy-containing or standard chow)

was also found, $F(2,84)=6.49$, $p<0.005$. Among rats treated with gentamicin, those fed the standard chow diet demonstrated a lower incorporation of [^{3}H]-Thymidine than groups fed the casein- or the soy-containing diets (Fig. 1).

3.1.2 Gentamicin cortical levels

A significant overall main effect of time of injection was found for gentamicin accumulation into renal cortices, $F(1,42)=16.08$, $p<0.0005$. Rats injected with gentamicin at 1200 h had a lower accumulation of gentamicin in renal cortical cells than rats injected with gentamicin at 2400 h. The overall main effect of diet was also significant, $F(2,42)=3.45$, $p<0.05$, with greater cortical accumulation of gentamicin in rats fed the casein-containing diet compared to rats fed with the standard chow diet.

3.1.3 Renal function

For the level of serum creatinine, a significant interaction between time of injection, treatment and diet was found, $F(2,83)=5.39$, $p<0.01$. Among animals injected at 1200 h, rats fed the casein-containing diet had significantly higher serum creatinine levels than rats fed the soy-containing diet and both had significantly higher serum creatinine levels when injected with gentamicin than their respective control treated with saline. No difference among dietary groups was found between gentamicin- and saline-treated rats at 2400 h (Fig. 2).

For creatinine clearance, a significant interaction between treatment and diet was found, $F(2,80)=5.32$, $p<0.01$. With the casein- and the soy-containing diets, gentamicin

treatment resulted in lower creatinine clearance than saline treatment. In rats fed the standard chow diet, no such difference was found (Fig. 3).

A significant interaction between time of injection, treatment and diet was found for BUN status, $F(2,83)=7.58$, $p<0.001$. Rats treated with gentamicin and fed the casein-containing diet had significantly higher BUN when injected at 1200 h compared to 2400 h. Among rats injected at 1200 h and fed casein- and soy-containing diets, those injected with gentamicin demonstrated significantly higher BUN than those injected with saline. On the other hand, no such difference was found in rats injected at 1200 h and fed the standard chow diet nor in the three dietary groups treated with both gentamicin and saline at 2400 h (Fig.4).

Diuresis and urinary proteins measured in percentage of increase from baseline (Day 0) to the final value measured on the last day of treatment (Day 15) were not significantly affected by time of injection, diet and treatment.

3.1.4 Enzymuria

A significant overall main effect of treatment was found for the excretion of NAG presented in percent of increase from baseline, $F(1,79)=2.69$, $p<0.05$. The increments in NAG excretion of gentamicin-treated rats were significantly higher in comparison to the saline-treated rats (gentamicin: 287 ± 33.59 *versus* saline: 179.45 ± 24.69). No significant result was found for the excretion of βGal presented in percent of increase from baseline.

3.1.5 Nutritional status

A significant overall main effect of time of injection was found for serum albumin values, $F(1,83)=9.51$, $p<0.005$. Rats injected at 2400 h had lower serum albumin values than rats injected at 1200 h, although all values remained within the desired range of 28-44 g/L for normal protein status. A significant overall main effect of diet was also found, $F(2,83)=22.280$, $p<0.0001$. More specifically, Scheffe's Post-Hoc test revealed that rats fed the soy-containing diet had higher serum albumin levels than those fed the standard chow diet, but both of the latter dietary groups showed lower serum albumin levels than those of rats fed the casein-containing diet (standard chow: 36.03 ± 0.60 *versus* soy: 39.56 ± 0.74 *versus* casein: 42.42 ± 0.84 g/L).

3.2 Body weight

Body weights of rats measured throughout the experimental period are presented in Fig. 5. In groups injected at 1200 h, the interaction between day and treatment was significant, $F(14,588)=53.07$, $p<0.0001$. A prominent difference in body weights was found during the last 6 days of the treatment period (Days 10 to 15), with lower body weights in rats injected with gentamicin than in rats injected with saline.

Groups treated at 2400 h only showed intra-individual variations in term of body weights with a significant main effect of day, $F(14,588)=24.75$, $p<0.0001$.

3.3 Food intake

3.3.1 24-h food intake

A significant interaction between day, treatment, and diet was found for total food intake (g) over 24-h in rats treated at 1200 h, $F(28,588)=2.04$, $p<0.05$. Food intake of rats treated at 1200 h with gentamicin was lower than that of saline-treated rats during the entire treatment period (Days 6 to 15) (Fig. 6). Interestingly, throughout the baseline and the treatment periods, but on Day 14, both gentamicin- and saline-treated groups fed the standard chow diet demonstrated no significant difference in the 24-h food intake. A suitable adaptation was demonstrated during the baseline period since there was no difference between dietary groups on Days 1 to 6 inclusively. In groups fed the casein-containing diet, rats injected with gentamicin demonstrated lower 24-h food intake than rats injected with saline on Days 9 to 15. Similarly, for groups fed the soy-containing diet, rats injected with gentamicin demonstrated lower 24-h food intake than rats injected with saline on Days 11 to 15. In the three dietary groups injected with gentamicin, 24-h food intake was comparable during the first half of the treatment period (Days 6 to 10), while the last portion of the treatment (Days 12 to 15) showed that the groups fed the casein- and soy-containing diets had similar 24-h intakes but significantly lower than the group fed the standard chow diet. During most of the treatment period (Days 7 to 15), each dietary group of rats injected with saline ate comparable amount of food over 24-h.

In rats treated at 2400 h, a significant interaction between day and treatment was found for the total food intake over 24-h, $F(14,588)=5.88$, $p<0.0001$. Food intake of rats

treated at 2400 h with gentamicin was lower than that of saline-treated rats during almost the entire treatment period (Days 7 to 15) (Fig. 6). A significant interaction between days and diet was also found, $F(28,588)=1.78$, $p<0.05$. During most of the treatment period (Days 8 to 15), the groups fed the casein- and soy-containing diets had 24-h intakes that were similar but significantly lower than in the group fed the standard chow diet.

3.3.2 12-h food intake measured during the light period

The 12-h food intake measured during the light period in groups treated at 1200 h was significantly affected by an interaction between day and treatment, $F(14,588)=7.58$, $p<0.0001$, with the 12-h light food intake of gentamicin-treated animals being lower than that of saline-treated animals during Days 7 to 15 of the treatment period. A significant interaction between day and diet was also found, $F(28,588)=2.83$, $p<0.005$, with the 12-h light food intake of rats fed the casein- and the soy-containing diets being lower than that of rats fed the standard chow diet on Days 12 to 15 (Fig.7).

A significant interaction between day and treatment was found for the 12-h food intake measured during the light period of the groups injected at 2400 h, $F(14,588)=5.04$, $p<0.0001$, with the 12-h light food intake of gentamicin-treated rats being lower than that of saline-treated rats on Days 11 to 15, which represent the second half on the treatment period. In addition, a significant interaction between day and diet was also found, $F(28,588)=2.73$, $p<0.0005$, with the 12-h light food intake of rats fed with the soy-containing diet being higher than that of rats fed with the casein-containing diet during the entire baseline period on Days 1 to 7 inclusively (Fig. 7).

3.3.3 12-h food intake measured during the dark period

The 12-h food intake measured during the dark period of the groups treated at 1200 h was significantly affected by an interaction between day, treatment, and diet, $F(28,588)=2.14$, $p<0.05$. During the entire treatment period (Days 6 to 15), food intake of rats treated with gentamicin was lower than that of saline-treated rats. In addition, no difference between dietary groups was found during Days 1 to 6 inclusively. In groups fed the casein- and the soy-containing diets, rats injected with gentamicin demonstrated lower 12-h dark food intake than rats injected with saline on Days 12 to 15. However, throughout the baseline and the treatment periods, both gentamicin- and saline treated groups fed the standard diet demonstrated no significant difference in the 12-h dark food intake. During the entire treatment period (Days 6 to 15), each dietary group of rats injected with saline ate comparable amount of food over the 12-h dark period. On the other hand, the three dietary groups injected with gentamicin had comparable 12-h dark food intake during the first half of the treatment period (Days 6 to 10), while over the last portion of the treatment (Days 12 to 15) the groups fed casein- and soy-containing diets had similar 12-h dark intakes but significantly lower than the group fed the standard diet (Fig. 8).

A significant main effect of diet was found in groups treated at 2400 h $F(2,42)=23.72$, $p<0.0001$, with 12-h dark food intake of rats fed the standard chow diet being higher than that of rats fed the casein- and the soy-containing diets. A significant interaction between day and treatment was demonstrated for the 12-h dark food intake of groups treated at 2400 h, $F(14,588)=3.52$, $p<0.001$, with the 12-h dark food intake of

gentamicin-treated rats being lower than that of saline-treated rats during almost all the treatment period (Days 7 to 12, and 14 to 15) (Fig. 8).

4.0 DISCUSSION

4.1 Nephrotoxicity parameters

This study shows that chronic gentamicin treatment at a daily dose of 40 mg/kg at 1200 or 2400 h for 10 consecutive days inflicted renal toxicity in female adult Sprague-Dawley rats, as shown by higher [^{3}H]-Thymidine accumulation into DNA of renal cortices and serum creatinine levels, lower creatinine clearance and marked increments in NAG excretion in urine, when compared to saline treatment. Temporal variations of gentamicin nephrotoxicity were also found, with higher [^{3}H]-Thymidine accumulation into DNA of renal cortices, serum creatinine levels and BUN, in rats treated with gentamicin in the middle of their resting period (at 1200 h) compared to those treated in the middle of their activity period (at 2400 h).

The aforementioned findings are reporting that time of the day at which gentamicin is administered influences nephrotoxicity induction. Originally, Nakano and Ogawa (1982) reported that lethal doses of gentamicin (285 mg/kg, s.c) killed more mice when injected in the middle of the resting period (1300 h) compared to other times of the day (0900, 1700, 2100, 0100, and 0500 h). Temporal variations of antibiotics induced were further confirmed by looking at nephrotoxicity parameters with different doses of an array of aminoglycosides (AG) in both male and female rats of various strains (Pariat *et al.*, 1984; Nakano 1990;

Beauchamp *et al.*, 1996; Julien *et al.*, 2000). Nephrotoxicity was found to be higher when various AG were administered in the middle of the resting period compared to the middle of the activity period (Yoshiyama *et al.*, 1992; Lin *et al.*, 1994;96; Beauchamp *et al.*, 1996; Julien *et al.*, 2000). In addition, a retrospective study by Prins *et al.*, (1997) conducted in hospitalized patients reported similar temporal variation patterns of gentamicin-induced nephrotoxicity, with a significantly higher incidence of renal dysfunction when gentamicin was administered during the resting period, between 0000 and 0730 h, compared to other times of the day.

In the present study, a lower intracortical accumulation of gentamicin was found in rats injected in the middle of their resting period, where the drug is supposed to be more toxic. However, other studies reported no significant difference in gentamicin intracortical accumulation between groups of rats injected in the middle of their resting (1300 h) or activity period (0100 h) (Lin *et al.*, 1994 a; Julien *et al.*, 2000). It is known that gentamicin concentration in renal proximal tubules does not correlate with it's nephrotoxicity but it is generally recognized that renal tubular necrosis is dose dependent (Bennett, 1989). High chronic doses of gentamicin are not necessary paralleled with high intracortical accumulation of gentamicin since cell necrosis (apoptosis) implies that dead cells are sloughed away, thus allowing the release of lysosomal gentamicin (Dallman *et al.*, 1974). Intracortical accumulation of gentamicin is also known to be a poor nephrotoxicity indicator for the management of aminoglycosides treatments.

Among rats injected with gentamicin in the middle of their resting period, those conditioned to the standard chow diet demonstrated lower [^{3}H]-Thymidine accumulation into DNA of renal cortices, BUN and serum creatinine levels and higher creatinine clearance compared to rats conditioned to the semi-purified casein- and soy-containing diets. Nephrotoxicity parameters including urine output (Beauchamp *et al.*, 1996), serum creatinine (Karzazi *et al.*, 1996; Julien *et al.*, 2000), creatinine clearance (Julien *et al.*, 2000) also demonstrated greater nephrotoxicity in rats injected with gentamicin in the middle of their resting period.

The presence of brush-border enzymes such as γ-glutamyl-transpeptidase (γ-GT) and lysosomal enzymes such as NAG and βGal in urine represents the primary clinical manifestation of renal toxicity and demonstrate that a chosen treatment induces toxicity. Excretion of NAG, presented in percent of increase from the baseline, was 62% higher with gentamicin treatment than with saline treatment which is consistent with results found by Yoshiyama *et al.*, 1992, who reported a 50% increased NAG from baseline in adult male Wistar rats chronically administered gentamicin at a dose of 60 mg/kg s.c. for 8 consecutive days. On the other hand, our results for βGal are discrepant with those obtained by others studies, since rats fed with casein demonstrated lower toxicity when injected with gentamicin compared to rats injected with saline (Pariat *et al.*, 1988; Yoshiyama *et al.*, 1992).

Karzazi *et al.*, (1998) had shown that nephrotoxicity indicators were influenced by macronutrient proportions of diets, with higher gentamicin cortical accumulation, βGal and

γ-GT in rats fed a lipid-rich diet (11.1% protein, 30% vegetable shortening and 10% soybean oil) than in those fed a protein-rich diet (55% casein, 3.3% lipid). In paralleled with this study, similar renal protection was confined by the standard chow diet containing low lipid level (18.1% mixed proteins and 4.5% lipid) compared to the two semi-purified 20% casein- and soy-containing diets (both with 10% safflower oil). A possible explanation could be that lipid-rich diets allow greater intracortical gentamicin accumulation as well as greater excretion of lysosomal enzymes. In the present study, lower casein percentage of 20% before and during gentamicin treatment may not have been sufficient to protect kidneys.

4.2 Body weight

No main effect of treatment nor of diet on body weight were found among rats treated in the middle of their activity period (2400 h). This clearly demonstrates that gentamicin administration during the activity period has not only a protective effect against renal toxicity but permits body weight maintenance, which is also important for gentamicin nephrotoxicity management. In contrast, during the last 6 days of the treatment period, body weights of rats treated with gentamicin in the middle of their resting period (1200 h) decreased when compared to saline-treated groups. Similarly, adult female Sprague-Dawley rats chronically administered with gentamicin (80 mg/kg, i.p.) at 1300 or 0100 h for 5 consecutive days displayed decreased body weights on the last day of the treatment period (Julien *et al.*, 2000). However, adult female Sprague-Dawley rats given an acute dose of gentamicin (150 mg/kg, i.p.) (time of injection not mentioned) did not show body weight

variations in the following three days (Karzazi *et al.*, 1998). In the present study, the fact that body weight remained comparable between groups may indicate that rats' dietary intake was adequate to meet their needs for energy balance.

4.3 Food Intake

An overall decrease in 24-h food intake occurred over the last 6 days of gentamicin treatment, regardless of time of administration. It was also found that 24-h food intake of gentamicin-injected rats was altered by diet composition, with higher food consumption in rats fed the standard chow diet compared to those fed casein- and soy-containing diets. Our findings concur with those of Karzazi *et al.*, (1998) who found that an acute gentamicin treatment (150 mg/kg, i.p.) at (time of injection not given) induced a significant reduction in caloric consumption one and three days post-injection in female Sprague-Dawley rats fed a lipid-rich diet containing 30% vegetable shortening and 10% soybean oil, compared to rats fed a protein-rich diet containing 55% casein. In addition, our results also concur with those found in a chronic study where gentamicin treatment (80 mg/kg/day, i.p.) at 1300 and 0100 h for 5 consecutive days resulted in a decrease in 24-h intake of standard chow (Julien *et al.*, 2000). Decreased total 24-h food intake by gentamicin treatment was significant and consistent among groups of rats regardless of the time of injection. Thus, in rats fed the casein- and the soy-containing semi-purified diets, the reduction of food intake by gentamicin could have contributed to aggravate renal toxicity and a consequent progression in renal toxicity could have decreased food intake. The presence of food was shown to be an important protective factor against renal toxicity of gentamicin. Indeed, Beauchamp *et*

al. (1996) have shown in normally fed (i.e., standard chow and water available *ad libitum* throughout the experiment) and fasted (i.e., only water available during a 12-h fast before and a 24-h fast after treatment) adult female Sprague-Dawley rats treated with an acute dose of gentamicin (150 mg/kg, i.p.), that fasted rats were more susceptible to renal toxicity. Therefore, it was suggested that dietary intake during the active period of rats is of crucial importance for the temporal modulation of gentamicin-induced nephrotoxicity.

A decrease in 12-h light and 12-h dark food intakes was observed in gentamicin-treated rats, regardless of the administration schedule. Interestingly, it is known that the nocturnal activity period of rats is the time at which they consume the greatest amount of food in order to meet their food requirements (Zucker, 1971), and this was also observed in the present experiment. Among rats injected at 2400 h, the 12-h light food intake was higher in animals fed the soy-containing diets, while the 12-h dark food intake was higher in those fed the standard chow diet compared to other dietary groups. This study report that rats injected at 1200 h and fed the standard chow diet had larger 12-h light and dark food intakes than rats fed the semi-purified casein- and soy-containing diets. Thus, notwithstanding the importance of maintaining adequate food consumption, one has to consider the food composition itself. Although few investigations were performed on dietary macronutrient properties and the role of dietary proteins, a study looked at the effect of protein restriction in adult male Sprague-Dawley rats fed *ad libitum* with either a standard Oxoid 18% protein diet or a low 5% protein diet (protein source and adaptation period not mentioned) and treated with a daily dose of gentamicin (120 mg/kg, i.p.) for 10

consecutive days. Whiting et al., (1988) reported a lower gentamicin-induced toxicity in rats fed the 5% protein diet, as shown by greater creatinine clearance, decrease in NAG activity and less marked histological changes compared to rats fed the 18% protein diet. In contrast, Beagle dogs adapted for 21 days to diets containing either 9.4%, 13.7% or 27.3% protein (protein source not mentioned) showed a higher creatinine clearance and urinary excretion of protein with the 27.3% protein diet compared to dogs fed the 9.4% protein diet at the end of their adaptation period. Thereafter, dogs were injected with gentamicin (10 mg/kg, i.m.) every 8 hours for 8 consecutive days, which resulted in more severe proximal tubular necrosis in dogs fed the 13.7% compared to those fed the 27.3% protein diet (Grauer *et al.*, 1994). Studies from Andrews and Bates (1987, 1988), examined the effect of dietary protein on renal function, both prior and during a 6 days gentamicin treatment (150 mg/kg, s.c.) in adult male Sprague-Dawley rats fed a Ralston Purina Company purified test isocaloric diets (casein being the protein source) with either low-protein (5%), normal-protein (20%) or high-protein (60%) contents. The first injection was administered after a 10 days conditioning period to dietary regimens, thereafter some rats conditioned to the 20% protein diet were switched to either the 5 or the 60% protein diets, and some of the rats conditioned to the 60% protein diet were switched to the 5% protein diet. The results showed that dietary protein content of the diet both prior and following gentamicin administration can affect renal function, histology and survival. Uptake of gentamicin in renal cortices decreased as dietary protein percentage in the diet increased prior to gentamicin treatment. Interestingly, rats conditioned to a 60% dietary protein diet and

switched to a 5% dietary protein diet demonstrated improvements in both mortality rate and renal function while rats switched from normal to high dietary protein showed a drastic increased in mortality rate.

Since the above studies were conducted in different animal species, using different percentages and sources of macronutrients, the protective effect of food against gentamicin nephrotoxicity can be attributed to macronutrient composition. Moreover, a clear relationship exists between phospholipidosis and nephrotoxicity, but it is still unclear how macronutrients act to alleviate gentamicin-induced nephrotoxicity. A fairly acidic environment favors electrostatic interactions between gentamicin and the negatively charged phospholipids. It is known that ionisation degree is altered by surrounding pH. Fresh urine is slightly acidic when an acid-residue diet is consumed and, following a meal, urine becomes more alkaline as gastric acid is secreted into the stomach, while high protein diets from meat cause persistently acidic urine (Tilkian, 1995). This might be a plausible mechanism for the protective effect of food. In addition, it has been suggested that varying the source of dietary protein may affect vasoconstrictor and/or vasodilatory substances secreted by the kidney (Dworkin *et al.*, 1983).

A noticeable difference exists for the 24-h food intake during the treatment period with gentamicin according to the dietary regimens consumed by rats. The nutritive value of a dietary component such as protein can be characterized according to its digestibility in order to compare different sources of protein together. York *et al.*, (1998) evaluated the digestibility of casein and soy, and found that the mean percentage digestibility of crude

casein and soy were respectively 95.4 and 92.1%. Soy protein's lower digestibility can be due to incomplete essential amino acid composition with methionine being the limiting amino acid. However, the use of protein isolate in the present study contributed to improve the nutritive value. In the standard chow diet, the crude protein portion content was essentially a mixture of vegetable proteins with a digestibility of 79.9%. Neophobia cannot explain lower food intake of gentamicin-treated rats fed the semi-purified casein- and soy-containing diets compared to those fed the standard chow diet since rats from the three dietary groups had similar food intake patterns during the baseline period. Since our diets were isocaloric and contained similar percentage of macronutrients, there is evidence that our results are a genuine consequence of the dietary protein source and lipid content. In addition, serum albumin values within the normal range among all dietary groups were indicative of a similar nutritional status. It has been suggested that dietary proteins rich in sulfur amino acid had antioxidative properties against renal toxicity (Tateishi, 1990; Hunter and Grimble, 1997). The sulfur amino acid breakdown of our respective diets displayed marked differences in term of the levels of cysteine (casein: 0.72, soy: 0.22, and standard chow: 0.51 g/100g of diet) and methionine (casein: 0.58, soy: 0.22, and standard chow: 0.43 g/100g of diet), but does not seem to support the previous assumption.

When one has a closer look at the role dietary composition may play to protect against nephrotoxic effect of gentamicin, the absolute protein content might not be the only factor. Generally, lipid-rich diets are low in protein since one macronutrient has to be lowered to the detriment of others in the diet preparation. In the present study, the lipid

contents varied among diets, with semi-purified casein and soy diets containing 10% safflower oil were associated with increased renal toxicity, whereas the non-purified standard chow diet containing 4.5% crude fat was found to be more protective than the former diets. It was found that a lipid-rich diet, composed of 60% rat chow, 30% vegetable shortening and 10% soybean oil exacerbated renal toxicity in rats injected with an acute dose of gentamicin (150 mg/kg, i.p.) at 0100 h, 0700 h and 1900 h but not at the peak of toxicity (at 1300 h), when compared to a 55% casein diet which showed a protective effect against renal toxicity (Karzazi *et al.*, 1998). More precisely, rats fed the lipid-rich diet had higher urinary βGal and γ-GT excretion in the first 24-h post-injection and higher intracortical accumulation of gentamicin. Consequently, it may be lipid rather than protein content of diets that modulate nephrotoxicity induced by gentamicin by increasing oxidative damage.

In conclusion, the main findings of this experiment support previous studies reporting that gentamicin-induced toxicity varies temporally, with a peak of nephrotoxicity when rats are injected in the middle of their resting period and a trough when injected in the middle of their activity period. We also support previous studies, which pinpoint that dietary composition alters gentamicin-induced nephrotoxicity, and that a mixed protein diet containing lower fat level protects against gentamicin-induced nephrotoxicity. The decreased food intake in rat administered with nephrotoxic doses of gentamicin and fed the semi-purified casein- and soy-containing diets observed in this experiment may have aggravated renal toxicity. Specific reasons for protective effect of food are not fully

understood, but mechanistic approaches should be undertaken using macronutrient composition of the diet to alter urinary pH enabling drug excretion, therefore limiting nephrotoxic effects induced by gentamicin. In addition, we also suggest that a lower fat content might halt or diminish phospholipidosis. Concrete applications to alleviate patient's gentamicin-induced nephrotoxicity could reside in administrations targeted in the middle of the day. Preventive and/or therapeutic dietary recommendations should be implemented to ensure adequate protein intake prior to gentamicin treatment while a low fat diet be maintained during the treatment period. Such recommendations would apply to both patients fed *per os* and for those receiving enteral and parenteral feedings, the latter being usually low in fat and adequate in protein.

Chapter 4

GENERAL CONCLUSION

This original study using adult female Sprague-Dawley rats chronically treated for 10 consecutive days with a nephrotoxic (not lethal) dose of gentamicin sulfate (40mg/kg/day, i.p.) at 1200 (middle of the resting period) or 2400 h (middle of the activity period), supports previous findings reporting that gentamicin-induced renal toxicity varies temporally, with a peak of nephrotoxicity when the drug is administered in the middle of the resting period and a trough of nephrotoxicity when administered in the middle of the activity period.

We observed that gentamicin reduced both the 12-light and 12-dark food intakes of rats leading to a significant decreased in total 24-h food consumption when compared to saline-treated rats. This study also shows that dietary composition alters gentamicin-induced nephrotoxicity, since the 18.1% mixed protein and the 4.5% fat content of the standard chow diet was more protective compared to the 10% fat semi-purified 20% of casein- and soy-containing diets. To our knowledge, two explanations can be provided to elucidate this finding. The first explanation resides in the fact that rats fed the semi-purified casein- and soy-containing diets significantly decreased their food consumption during the gentamicin treatment period. It is known that the presence of food with concurrent gentamicin treatment can decrease the induction of nephrotoxicity (Beauchamp *et al*, 1996;1997; Julien *et al*., 2000). Thus, if rats eat less food, they should be inflicted with greater nephrotoxicity, and consequently rats suffering from nephrotoxicity will eat less and

so on. The second explanation resides in the fact that an exacerbation in renal toxicity could have been related to the lipid content of the diets. The 10% fat level of semi-purified casein- and soy-containing diets was more than twice the 4.5% fat contained in the standard chow diet, which was possibly enough to favor renal toxicity considering that rats usually eat 10% of their body weight on a daily basis. A high dietary fat intake was also found to exacerbate gentamicin-induced renal toxicity (Karzazi *et al.*, 1998).

Notwithstanding the fact that a protein-rich diet, made of 55% casein mixed with 45% standard chow, confined protective effects against nephrotoxicity (Karzazi *et al.*, 1998), we could not reproduce those results using a 20% casein-containing semi-purified diet. It is possible that 20% casein was not sufficient to be protective, or that the presence of standard chow in the mixture contains a "key" ingredient capable to confine protective properties.

A limitation of this study resides in the different percentage of more than one macronutrient in the dietary regimens tested. Initially, we felt that the protective effect of a casein-rich diet (Karzazi *et al.*, 1998) was strong enough to base further studies on the protein source alone. Although, this experiment was reinforced by a double-controlled design in which each rat was used as its own control and a between-group comparison was made possible between the saline- and the gentamicin-treated groups, it was not possible to evaluate whether the protective effect was due to the protein source itself or if it was the low dietary lipid content that exacerbated nephrotoxicity. Therefore, we suggest that only

one macronutriment differ among diets while the others remain constant in order to pinpoint the effects.

Specific mechanisms explaining the protective effect of food are not fully understood, but mechanistic approaches have been suggested to which we add the possible effect that different macronutrient composition and percentage might alter urinary pH enabling drug excretion, thus limiting toxic effects.

Table 1: Dietary composition of the semi-purified protein-containing diets (dry weight, g/100 g of diet)

Ingredients*	Casein diet	Soy diet
Casein (high nitrogen, 95% protein)	20.00	-
Soy protein isolated (92% protein)	-	20.00
Sucrose	23.40	23.40
Cornstarch	35.15	35.15
Safflower oil	10.00	10.00
Cellulose, alphacel	5.00	5.00
AIN-76 salt mixture	5.25	5.25
AIN-76 vitamin mixture	1.10	1.10
Choline chloride	0.10	0.10

*Purchased from ICN Biomedicals (Aurora, OH)

Table 2: Macronutrients composition of diets

Diets	Macronutrients (g/100 g of diet)				Caloric density (kcal/g of diet)
	Proteins	Lipids	CHO	Fibers	
Casein-containing[1]	20	10	58.55	5.0	3.94
Soy-containing[1]	20	10	58.55	5.0	4.0
Standard chow[2]	18.1	4.5	57.30	3.4	4.1

[1] Semi-purified diets prepared in our laboratory
[2] Non-purified Chow 5075, Charles River Canada inc.

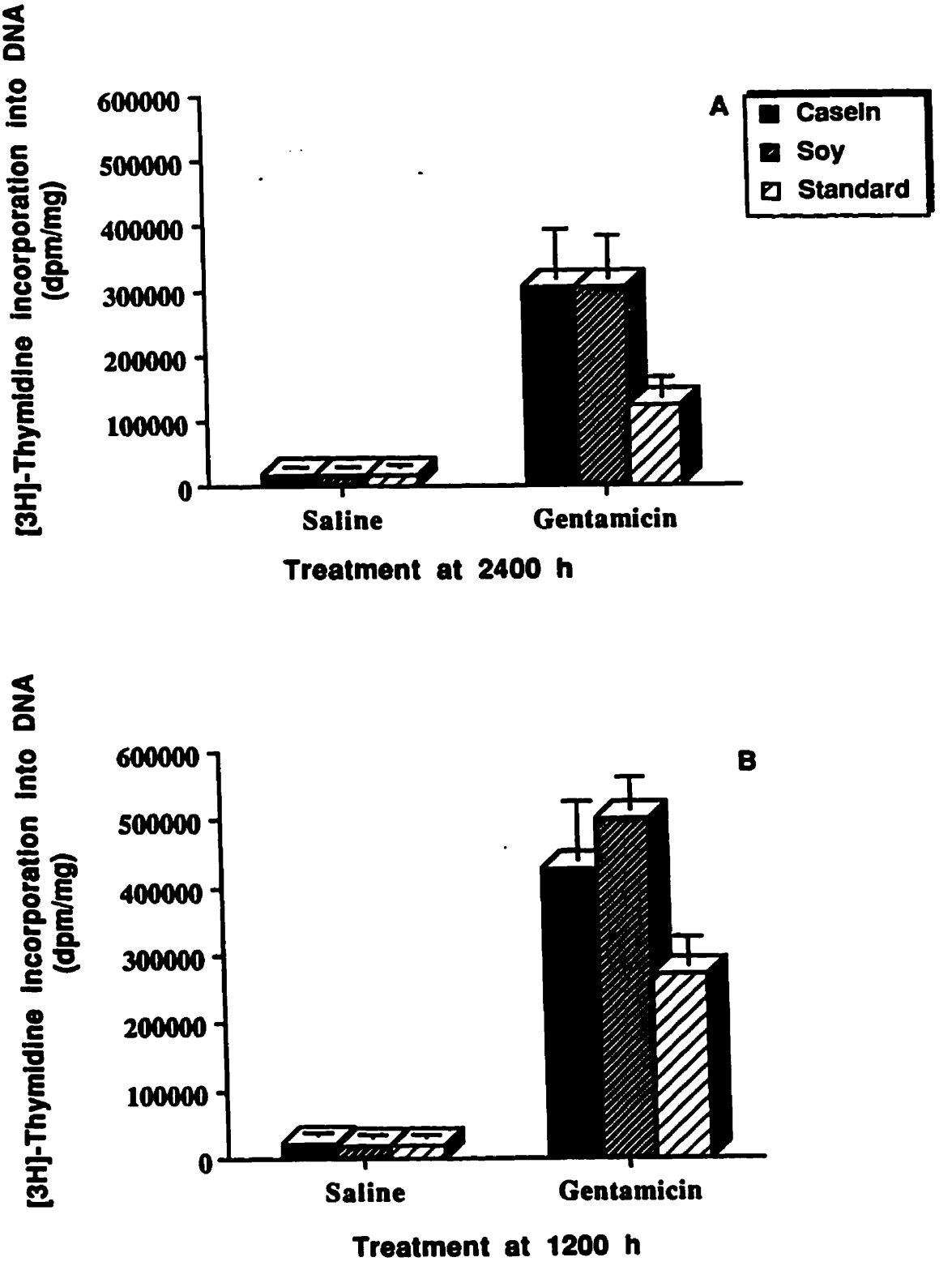


FIG. 1: The effect of treatment by diet on [^{3}H]-Thymidine incorporation into DNA in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM.

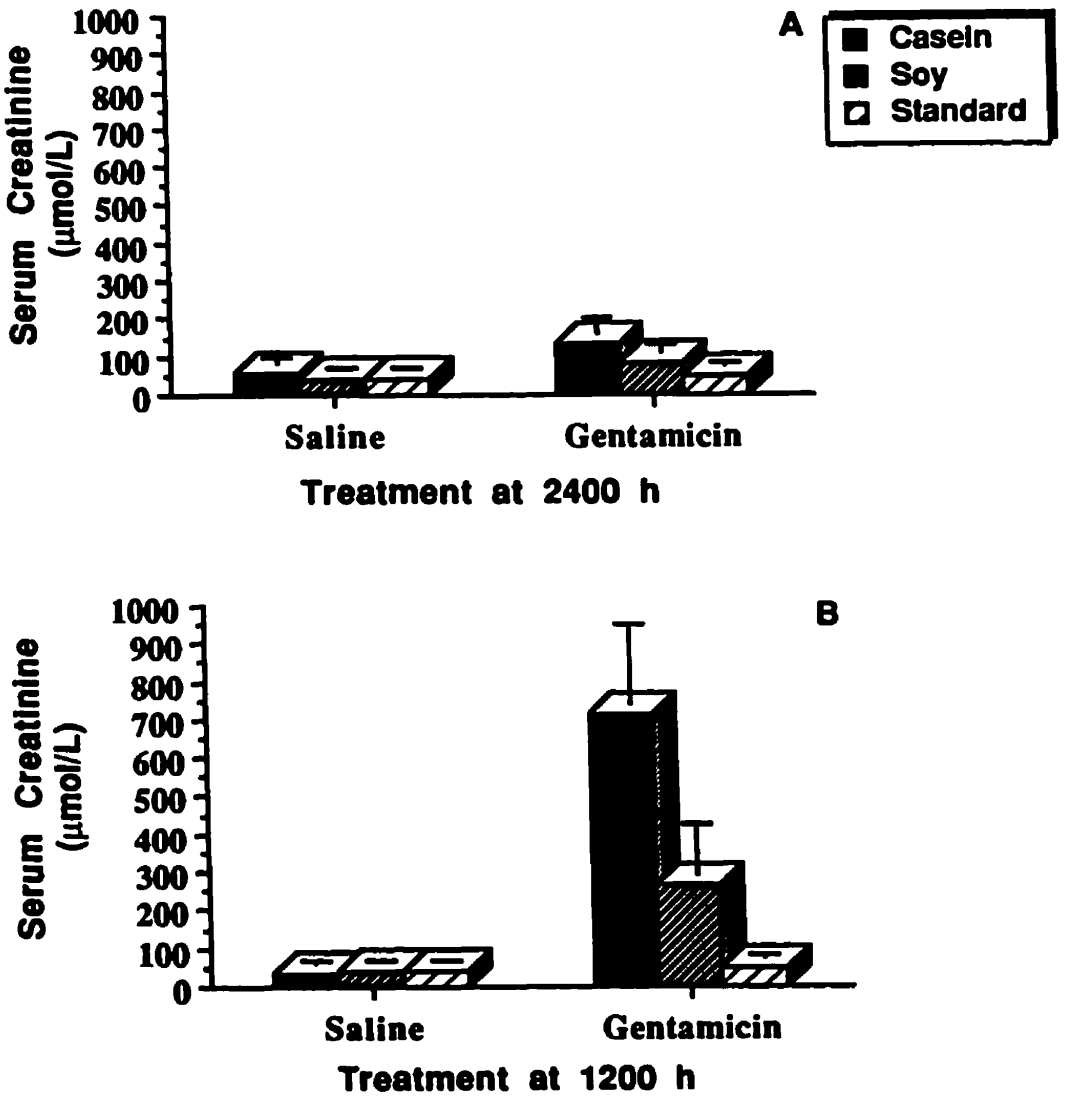
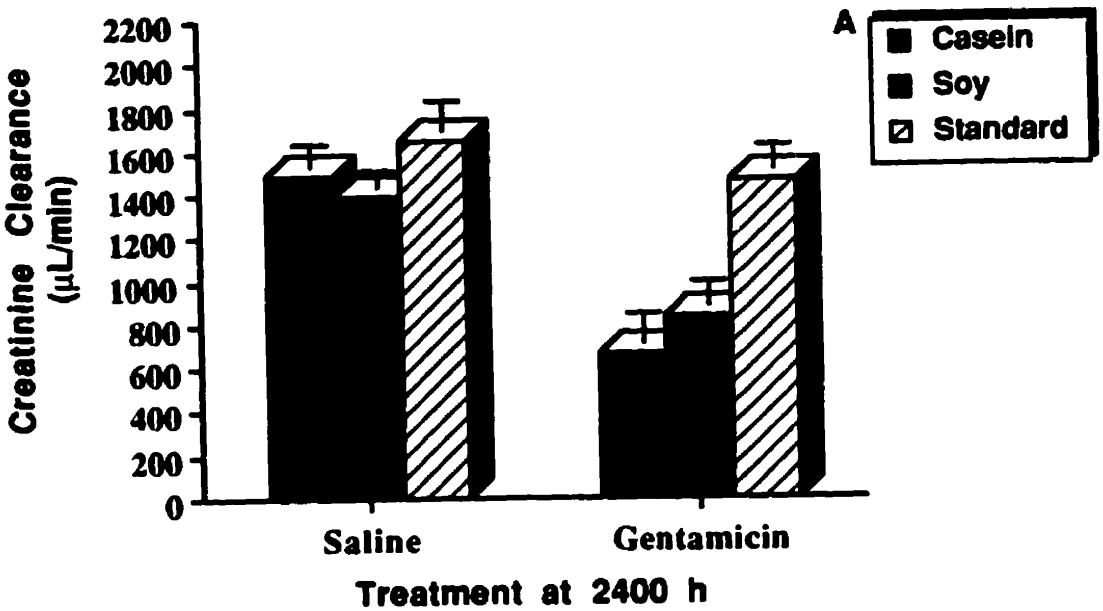


FIG. 2: The effect of treatment by diet on serum creatinine in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM.



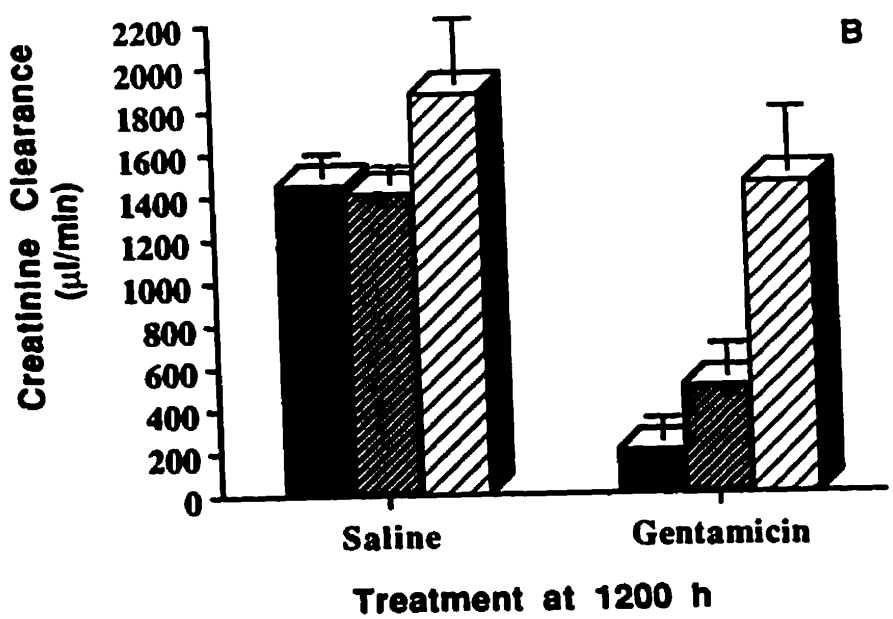


FIG. 3: The effect of treatment by diet on creatinine clearance in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM.

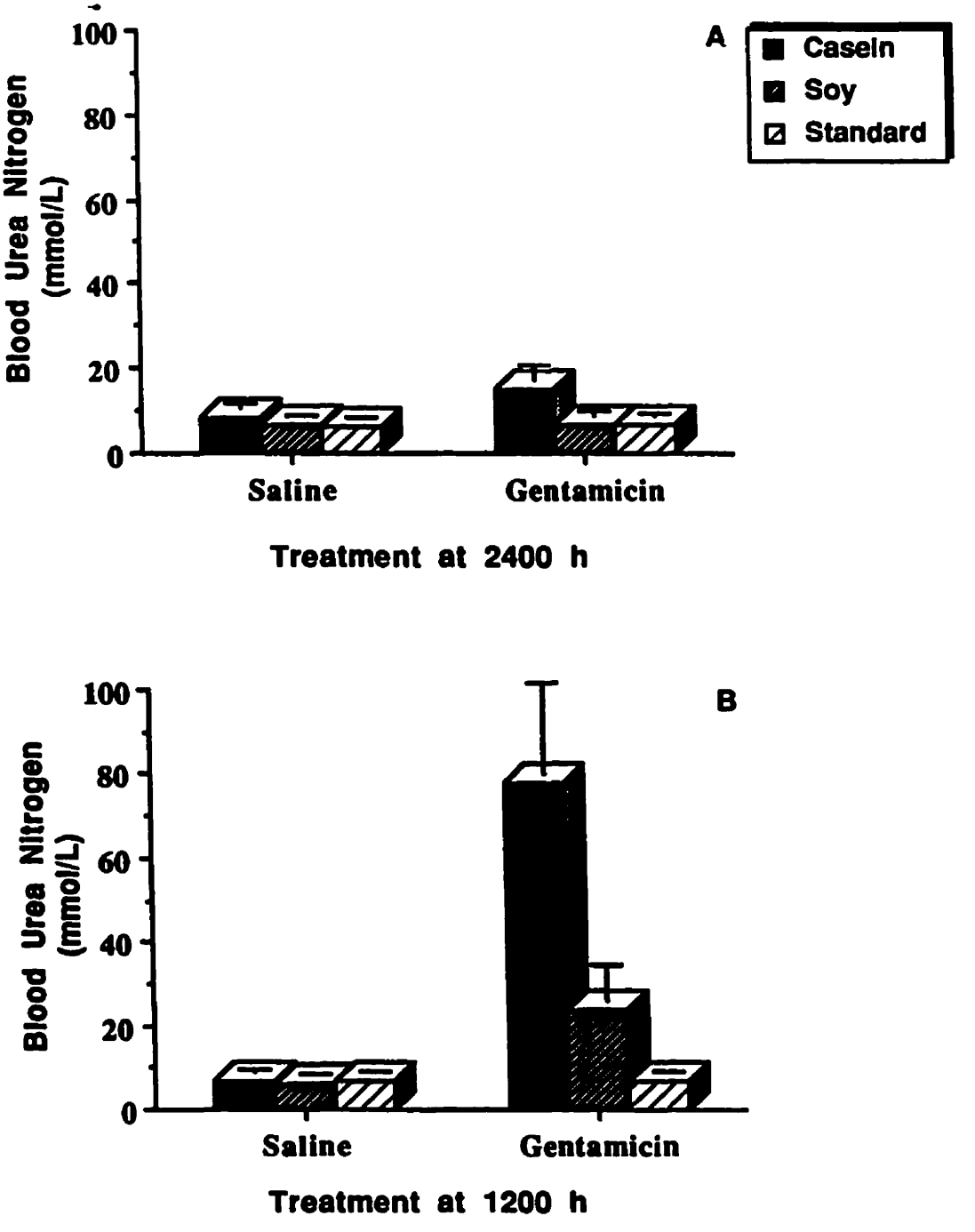


FIG. 4: The effect of treatment by diet on blood urea nitrogen in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM.

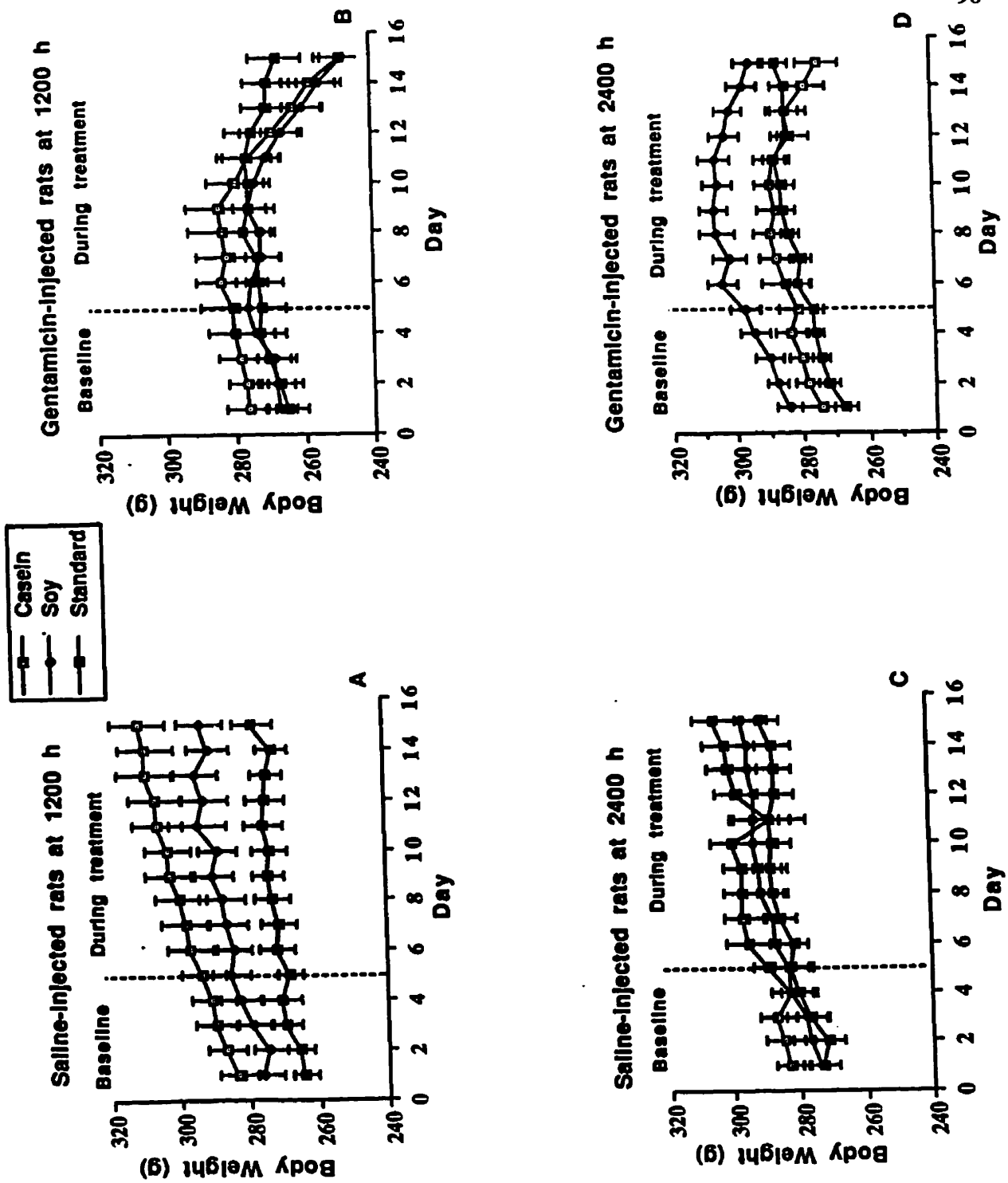


FIG. 5: Body weights of rats throughout the experimental period fed a casein, a soy-containing or a standard chow diet. Days 1 to 5 correspond to the baseline period, and Days 6 to 15 correspond to the treatment period where rats were treated with saline at 1200 h (A) or 2400 h (C) or gentamicin (40 mg/kg, i.p.) at 1200 h (B) or 2400 h (D).

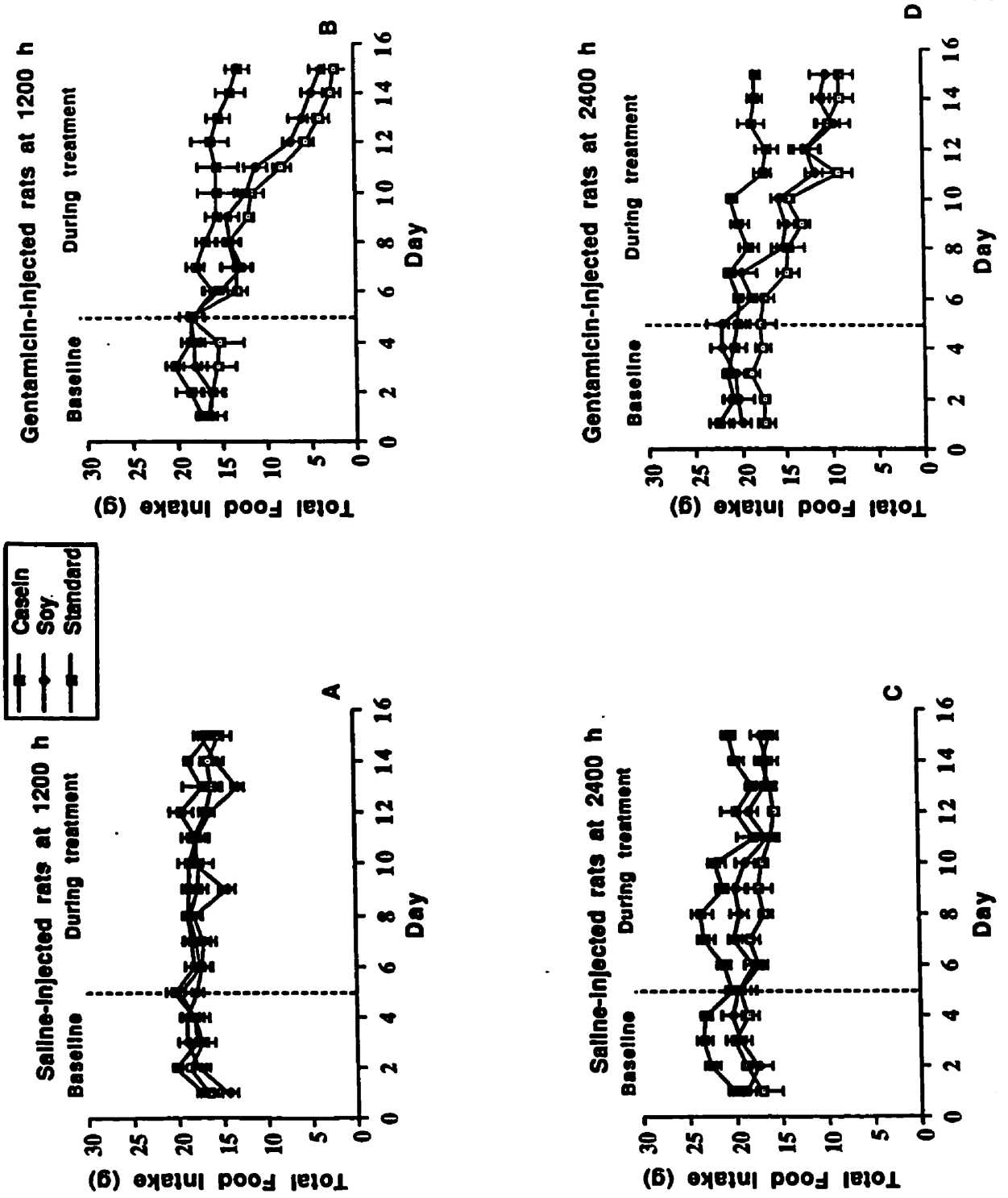


FIG. 6: Total food intake of rats throughout the experimental period fed a casein, a soy-containing or a standard chow diet. Days 1 to 5 correspond to the baseline period, and Days 6 to 15 correspond to the treatment period where rats were treated with saline at 1200 h (A) or 2400 h (C) or gentamicin (40 mg/kg, i.p.) at 1200 h (B) or 2400 h (D).

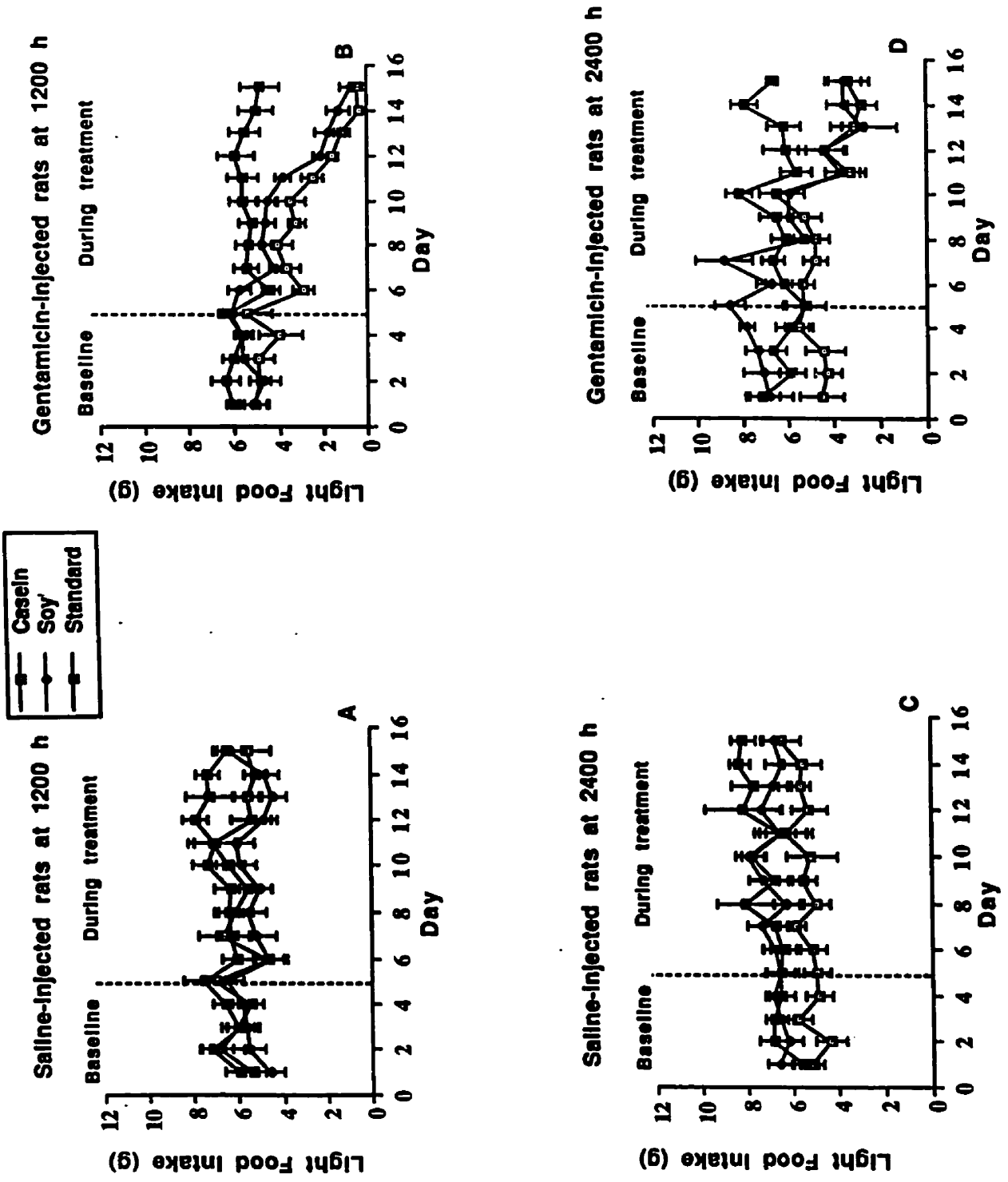


FIG. 7: 12-h light food intake of rats throughout the experimental period fed a casein, a soy-containing or a standard chow diet. Days 1 to 5 correspond to the baseline period, and Days 6 to 15 correspond to the treatment period where rats were treated with saline at 1200 h (A) or 2400 h (C) or gentamicin (40 mg/kg, i.p.) at 1200 h (B) or 2400 h (D).

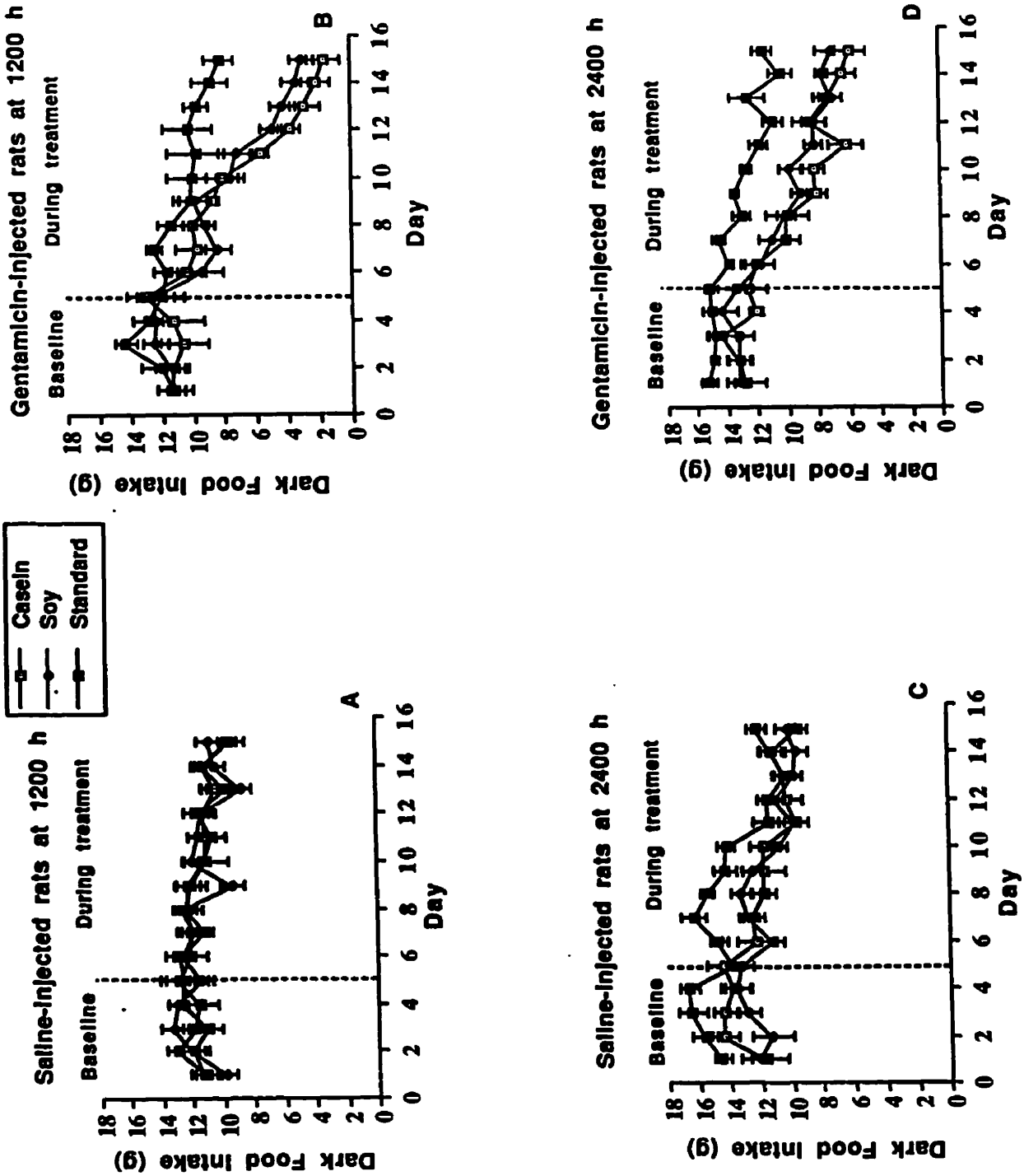


FIG. 8: 12-h dark food intake of rats throughout the experimental period fed a casein, a soy-containing or a standard chow diet. Days 1 to 5 correspond to the baseline period, and Days 6 to 15 correspond to the treatment period where rats were treated with saline at 1200 h (A) or 2400 h (C) or gentamicin (40 mg/kg, i.p.) at 1200 h (B) or 2400 h (D).

APPENDICES

TABLE A1. Main effects of factorial ANOVA[1] on nephrotoxicity parameters in rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

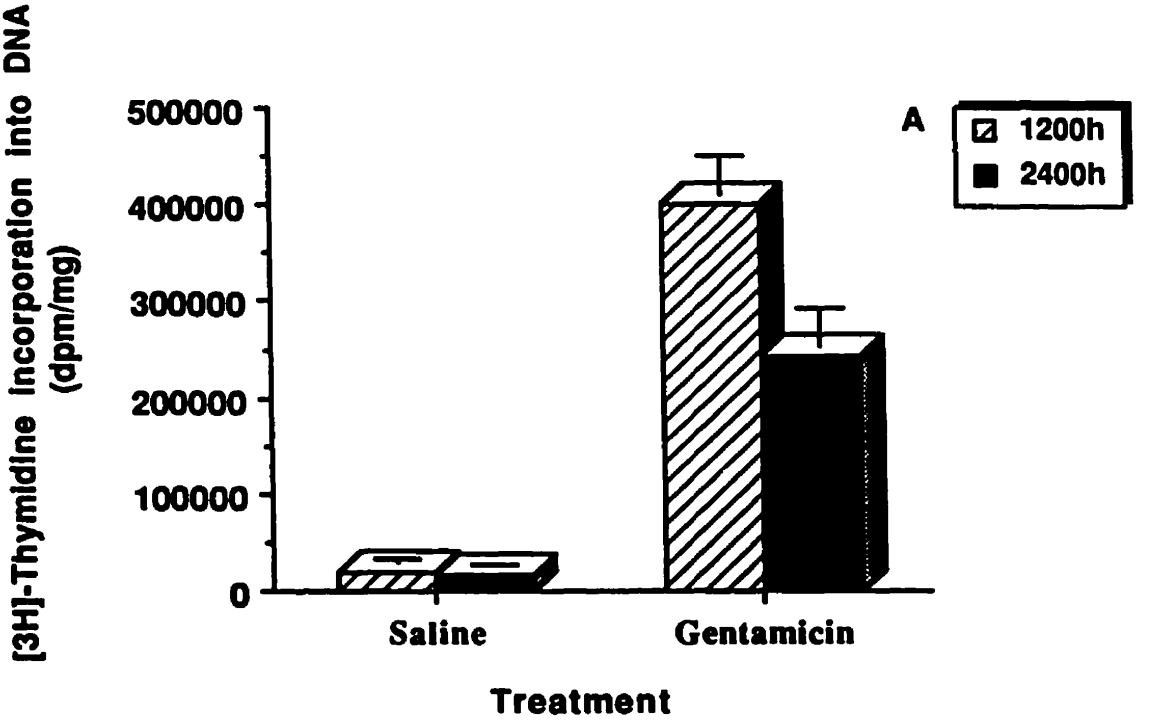
NEPHROTOXICITY PARAMETERS	MAIN EFFECTS					
	TIME OF INJECTION[2]		TREATMENT[3]		DIET[4]	
	F value	probability	F value	probability	F value	probability
[^{3}H]-thymidine incorporation	(1,84)=10.107	0.0021	(1,84)=145.12	0.0001	(2,84)=6.365	0.0027
Serum creatinine	(1,83)=11.018	0.0013	(1,83)=21.121	0.0001	(2,83)=8.511	0.0004
Creatinine clearance	(1,80)=1.106	0.2961	(1,80)=53.575	0.0001	(2,80)=19.913	0.0001
Blood urea nitrogen	(1,83)=13.928	0.0003	(1,83)=21.353	0.0001	(2,83)=12.609	0.0001
Serum albumin	(1,83)=9.508	0.0028	(1,83)=2.757	0.1006	(2,83)=22.280	0.0001
Gentamicin cortical levels	(1,42)=17.715	0.0001	-	-	(2,42)=6.613	0.0032
Diuresis (% baseline)	(1,83)=0.082	0.7758	(1,83)=1.496	0.2247	(2,83)=1.824	0.1679
βGal (% baseline)	(1,78)=0.232	0.6314	(1,78)=0.089	0.7661	(2,78)=0.164	0.8488
NAG (% baseline)	(1,79)=0.430	0.5138	(1,79)=6.693	0.0115	(2,79)=1.226	0.2990
Urinary proteins (% baseline)	(1,78)=0.169	0.6818	(1,78)=0.176	0.6756	(2,78)=0.374	0.6892

[1] ANOVA is restricted to the between-subject effects; a rat is a "subject".
[2] Time of injection main effect (1200 h or 2400 h).
[3] Treatment main effect (gentamicin or saline).
[4] Diet main effect.(casein, soy or standard chow).

TABLE A2. Interactions of factorial ANOVA[1] on nephrotoxicity parameters in rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

NEPHROTOXICITY PARAMETERS	INTERACTIONS							
	TIME OF INJECTION[2]* TREATMENT[3]		TIME OF INJECTION*DIET[4]		TREATMENT*DIET		TIME OF INJECTION* TREATMENT*DIET	
	F value	probability	F value	probability	F value	probability	F value	probability
[^{3}H]-thymidine incorporation	(1,84)=9.008	0.0035	(2,84)=0.156	0.8556	(2,84)=6.493	0.0024	(2,84)=0.192	0.8257
Serum creatinine	(1,83)=11.601	0.0010	(2,83)=4.638	0.0123	(2,83)=7.989	0.0007	(2,83)=5.389	0.0063
Creatinine clearance	(1,80)=3.324	0.0720	(2,80)=1.324	0.2717	(2,80)=5.322	0.0068	(2,80)=0.079	0.9240
Blood urea nitrogen	(1,83)=14.654	0.0002	(2,83)=6.822	0.0018	(2,83)=10.869	0.0001	(2,83)=7.578	0.0009
Serum albumin	(1,83)=0.851	0.3590	(2,83)=0.192	0.8258	(2,83)=4.077	0.0205	(2,83)=0.254	0.7763
Gentamicin cortical levels	-	-	(2,42)=1.157	0.3243	-	-	-	-
Diuresis (% baseline)	(1,83)=0.528	0.4696	(2,83)=0.128	0.8798	(2,83)=0.752	0.4744	(2,83)=0.009	0.9911
βGal (% baseline)	(1,78)=0.038	0.8468	(2,78)=0.749	0.4764	(2,78)=4.142	0.0195	(2,78)=2.372	0.1000
NAG (% baseline)	(1,79)=0.011	0.9224	(2,79)=0.183	0.8327	(2,79)=0.747	0.4769	(2,79)=0.665	0.5174
Urinary proteins (% baseline)	(1,78)=0.599	0.4412	(2,78)=0.628	0.5362	(2,78)=1.405	0.2515	(2,78)=0.690	0.5044

[1] ANOVA is restricted to the between-subject effects; a rat is a "subject".
[2] Time of injection main effect (1200 h or 2400 h).
[3] Treatment main effect (gentamicin or saline).
[4] Diet main effect (casein, soy or standard chow).



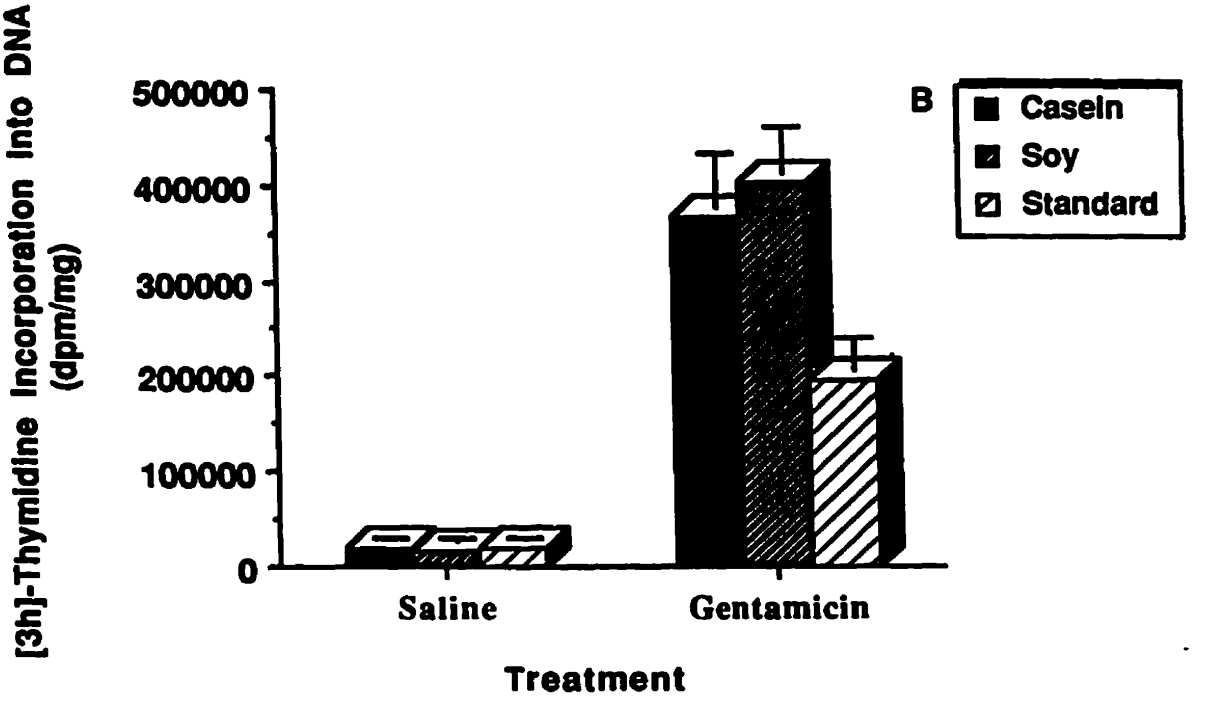


FIG. A1a: The effect of the time of injection by treatment (A) and of treatment by diet (B) on [^{3}H]-Thymidine incorporation into DNA of renal cortices of rats fed a casein-, a soy-containing, or a standard chow diet and treated for ten days with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or 2400 h. Data expressed as mean ± SEM. See Table A2 for significance.

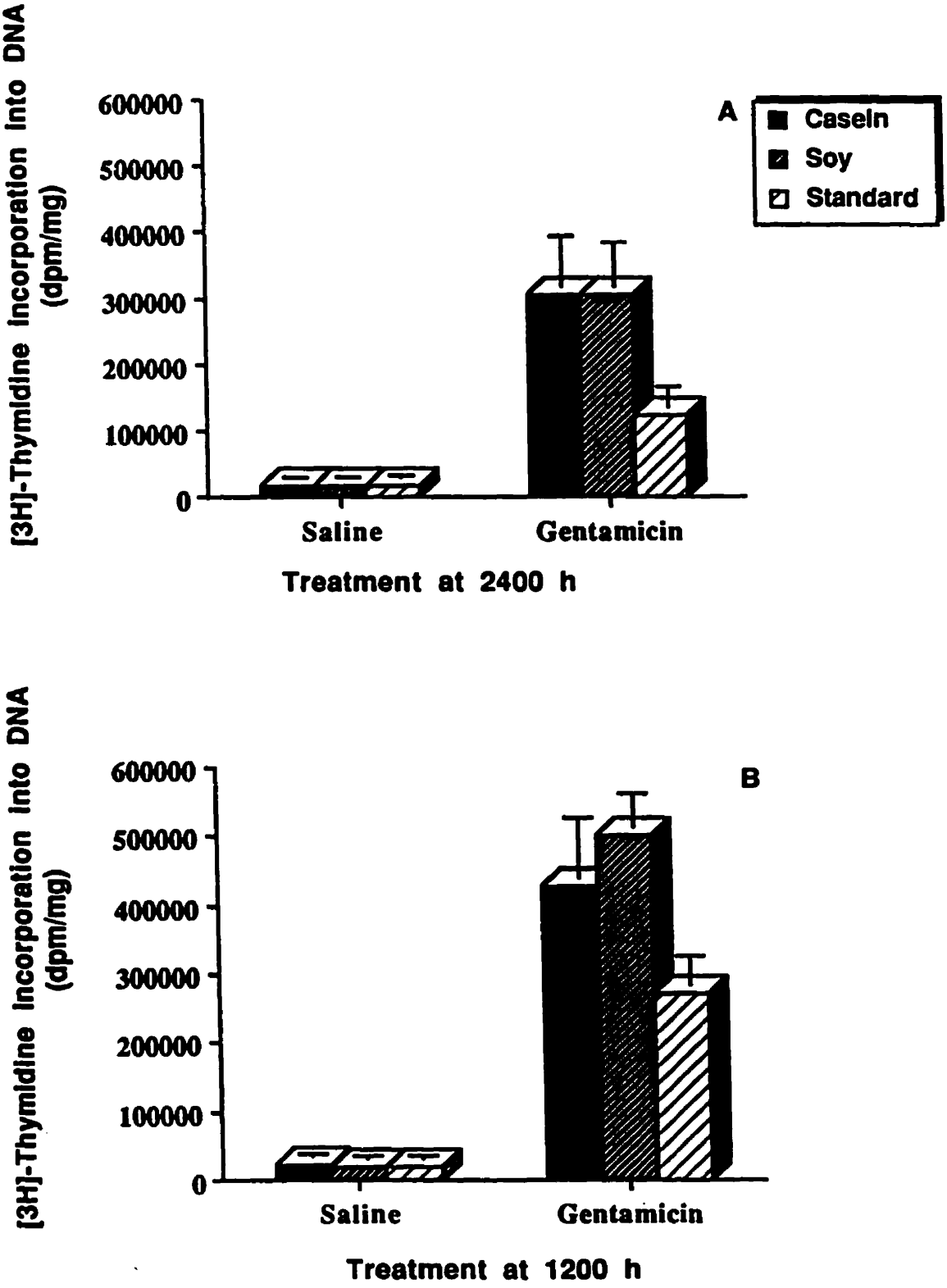


FIG. A1b: The effect of treatment by diet on [^{3}H]-Thymidine incorporation into DNA in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM. See Table A2 for significance.

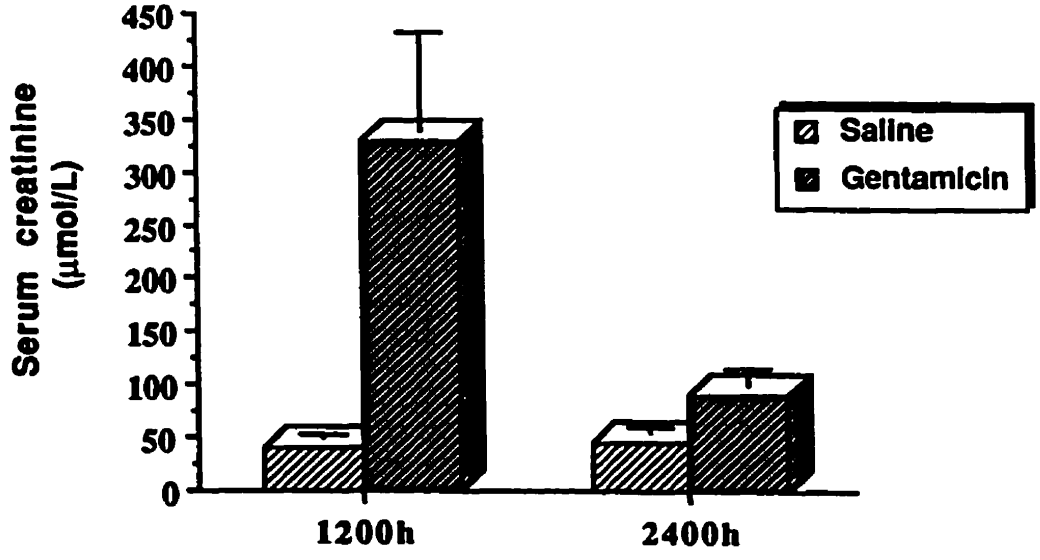


FIG. A1c: The effect of treatment on serum creatinine levels in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A1 for significance.

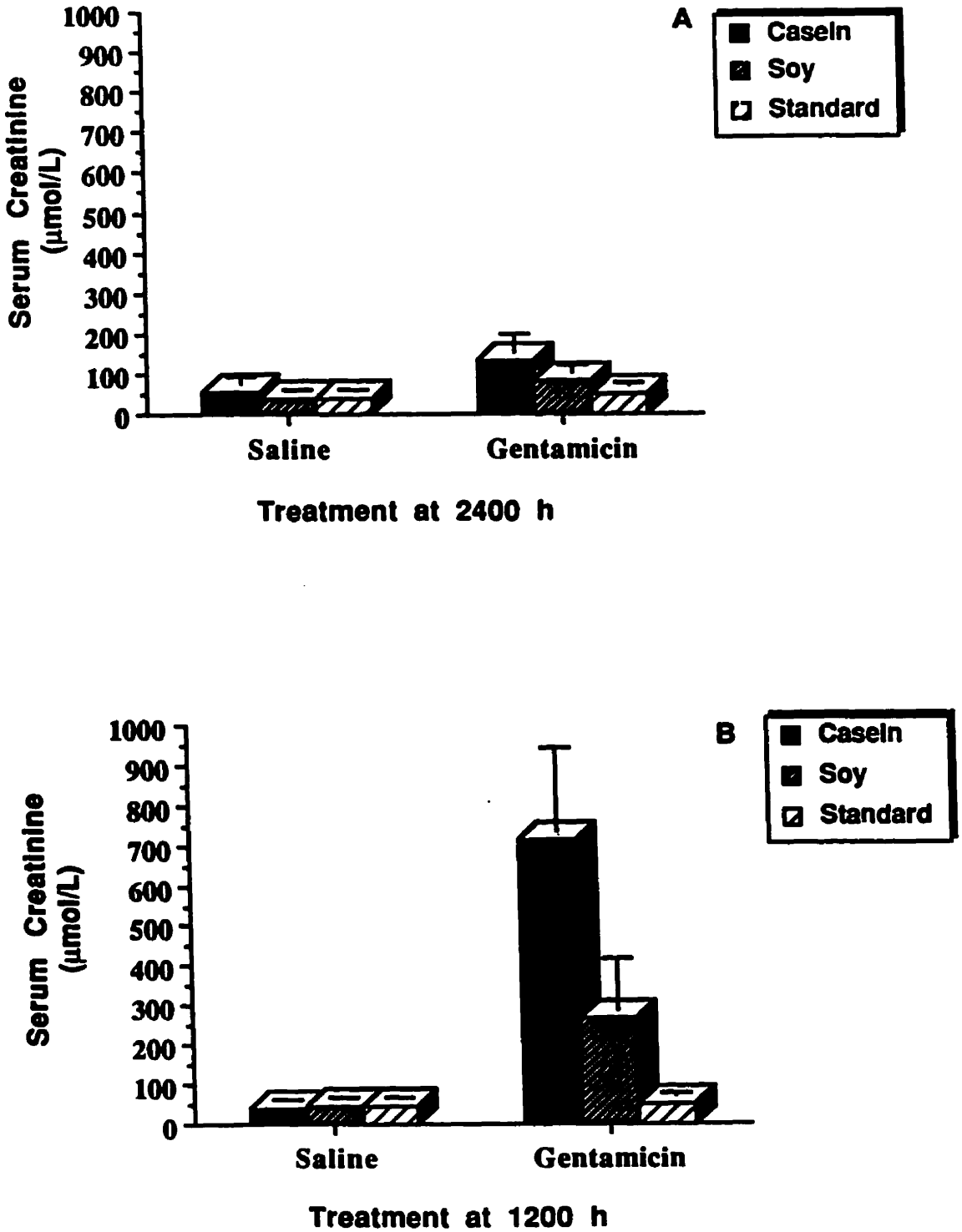


FIG. A1d: The effect of treatment by diet on serum creatinine levels in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) and at 1200 h (B). Data expressed as mean ± SEM. See Table A2 for significance.

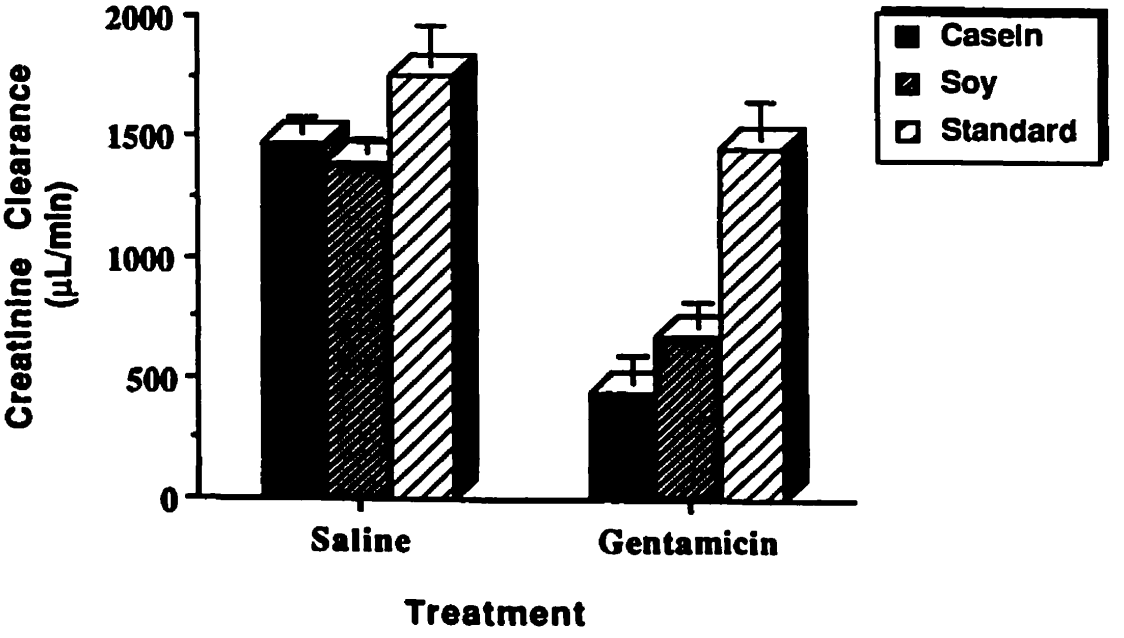
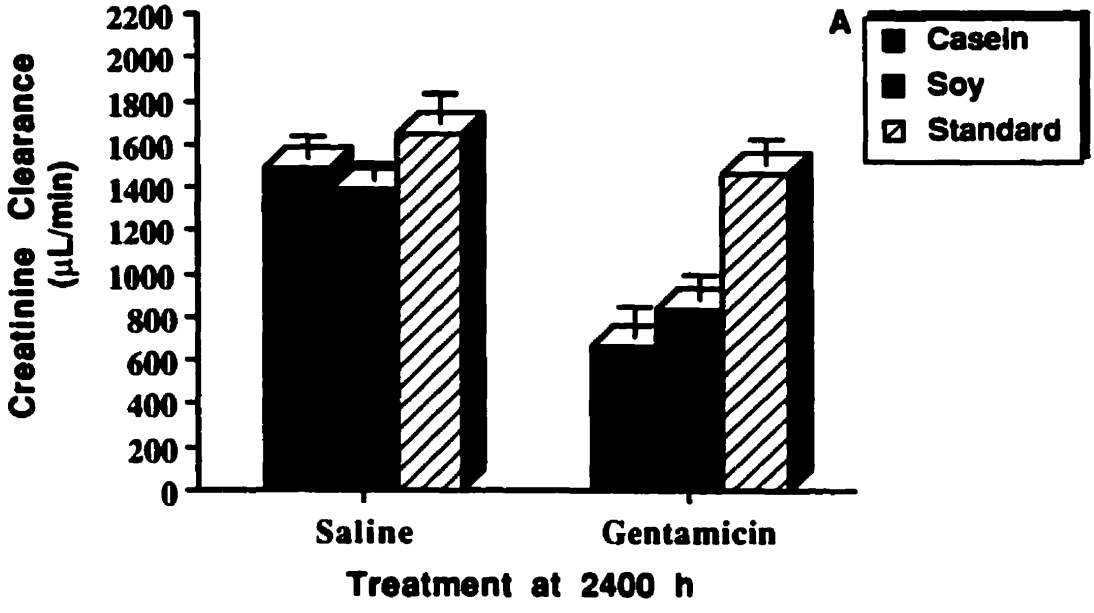


FIG. A1e: The effect of treatment by diet on creatinine clearance in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A2 for significance.



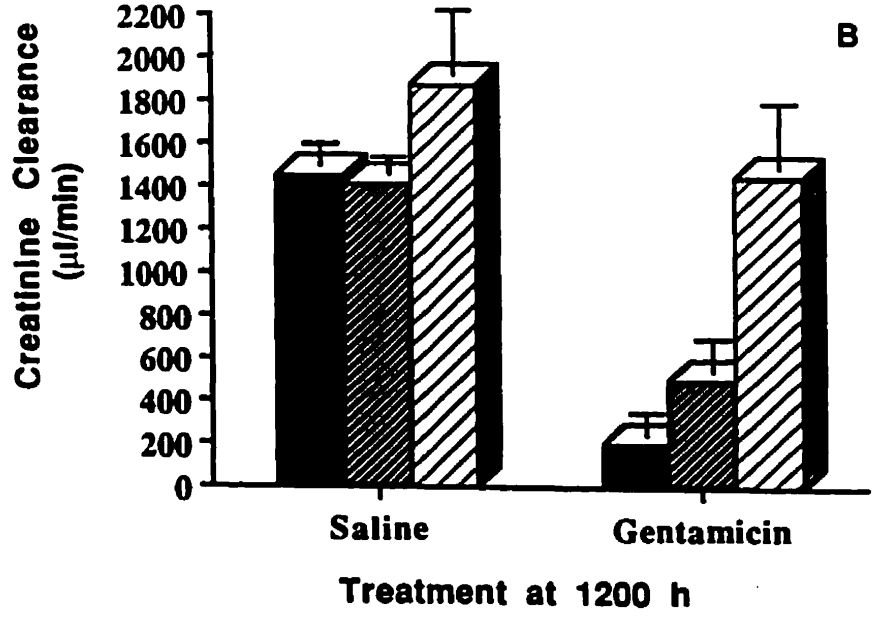


FIG. A1f: The effect of treatment by diet on creatinine clearance in rats fed a casein-, a soy-containing, or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM. See Table A2 for significance.

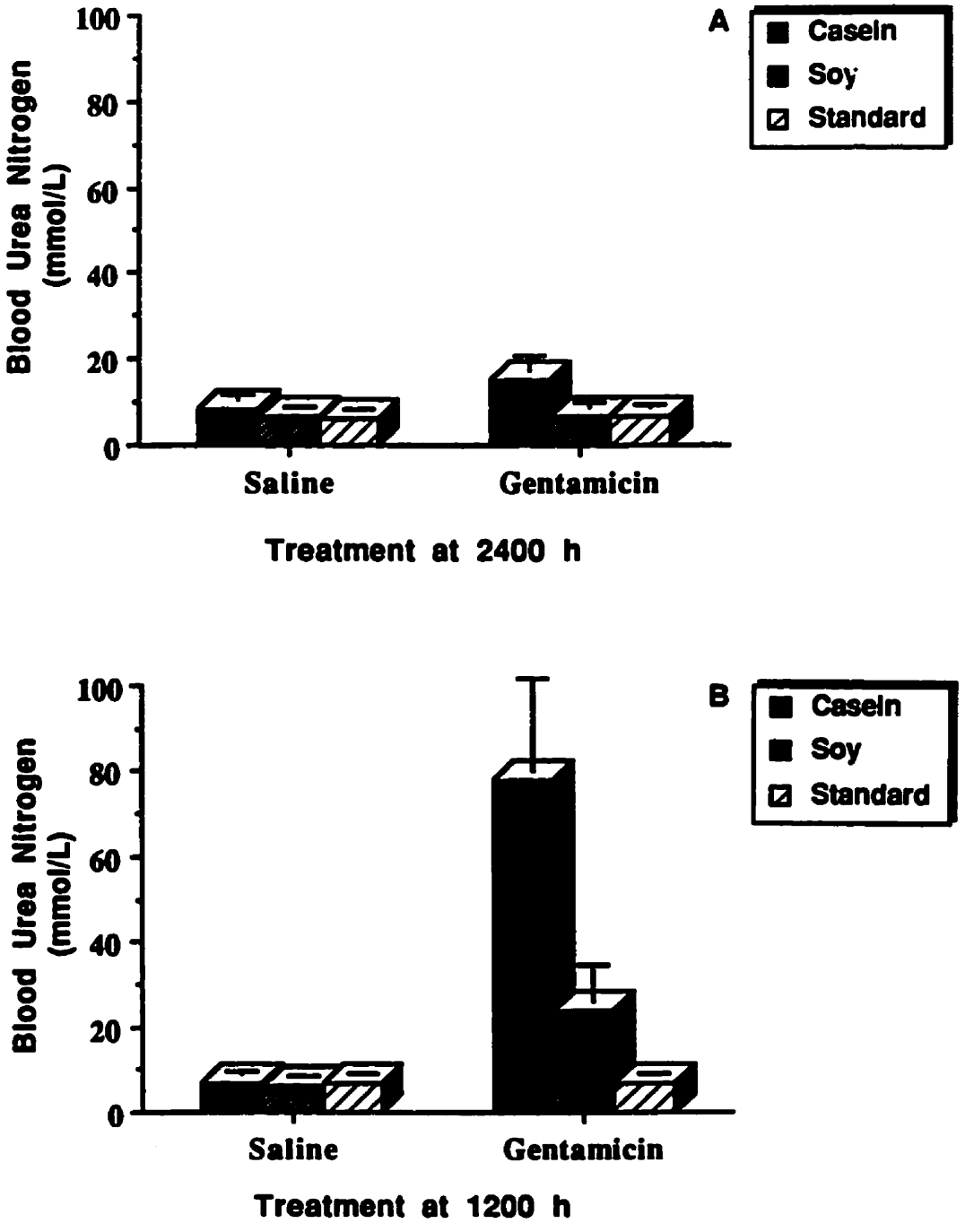


FIG. A1g: The effect of treatment by diet on blood urea nitrogen in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h (A) and at 2400 h (B). Data expressed as mean ± SEM. See Table A2 for significance.

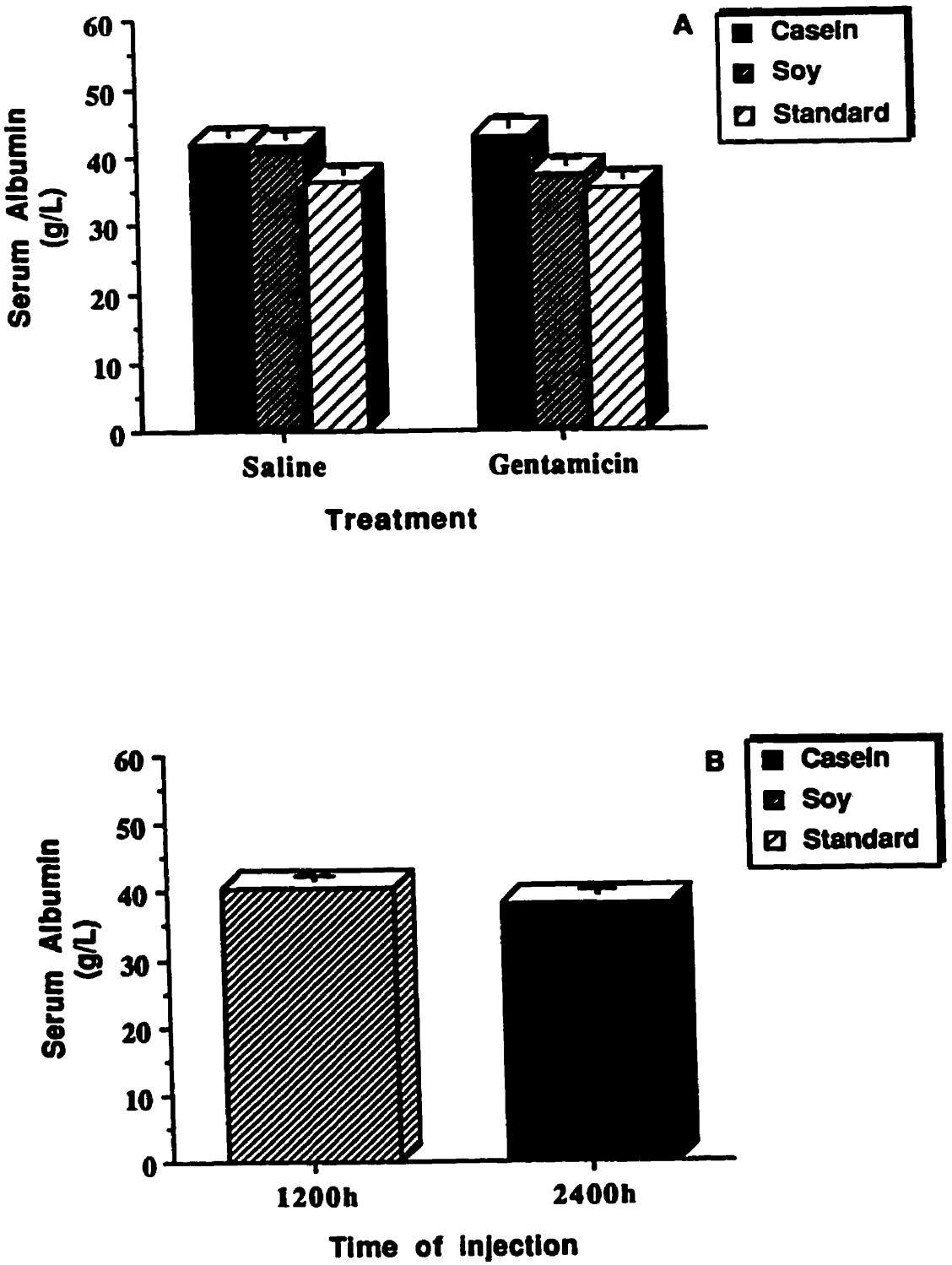


FIG. A1h: The effect of treatment by diet (A) and of time of injection (B) on serum albumin levels in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Tables A1 and A2 for significance.

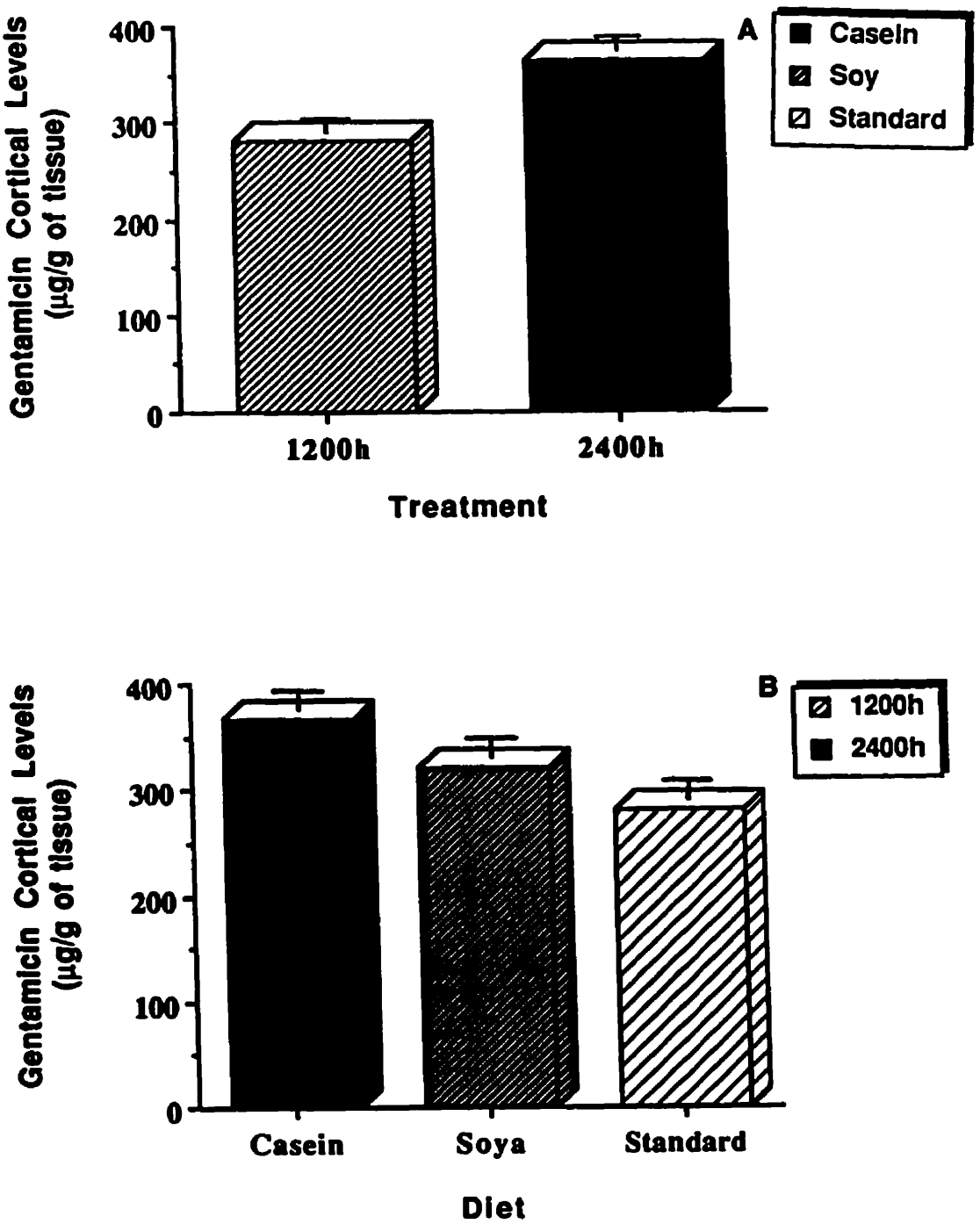


FIG. A1i: The effect of time of injection (A) and of diet (B) on gentamicin cortical levels in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A1 for significance.

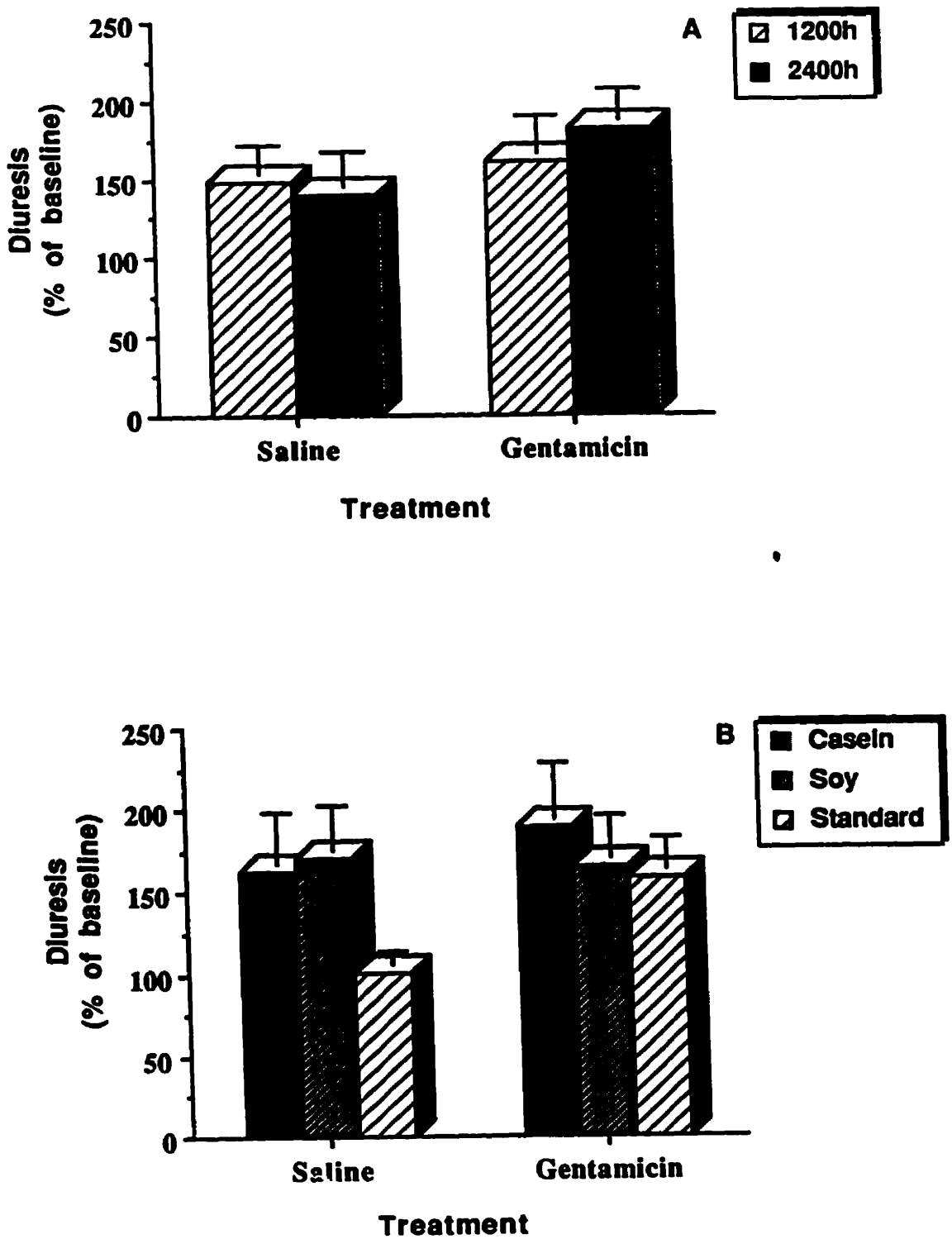


FIG. A1j: The effect of time of injection by treatment (A) and of treatment by diet (B) on diuresis measured in percent of baseline in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A2 for significance.

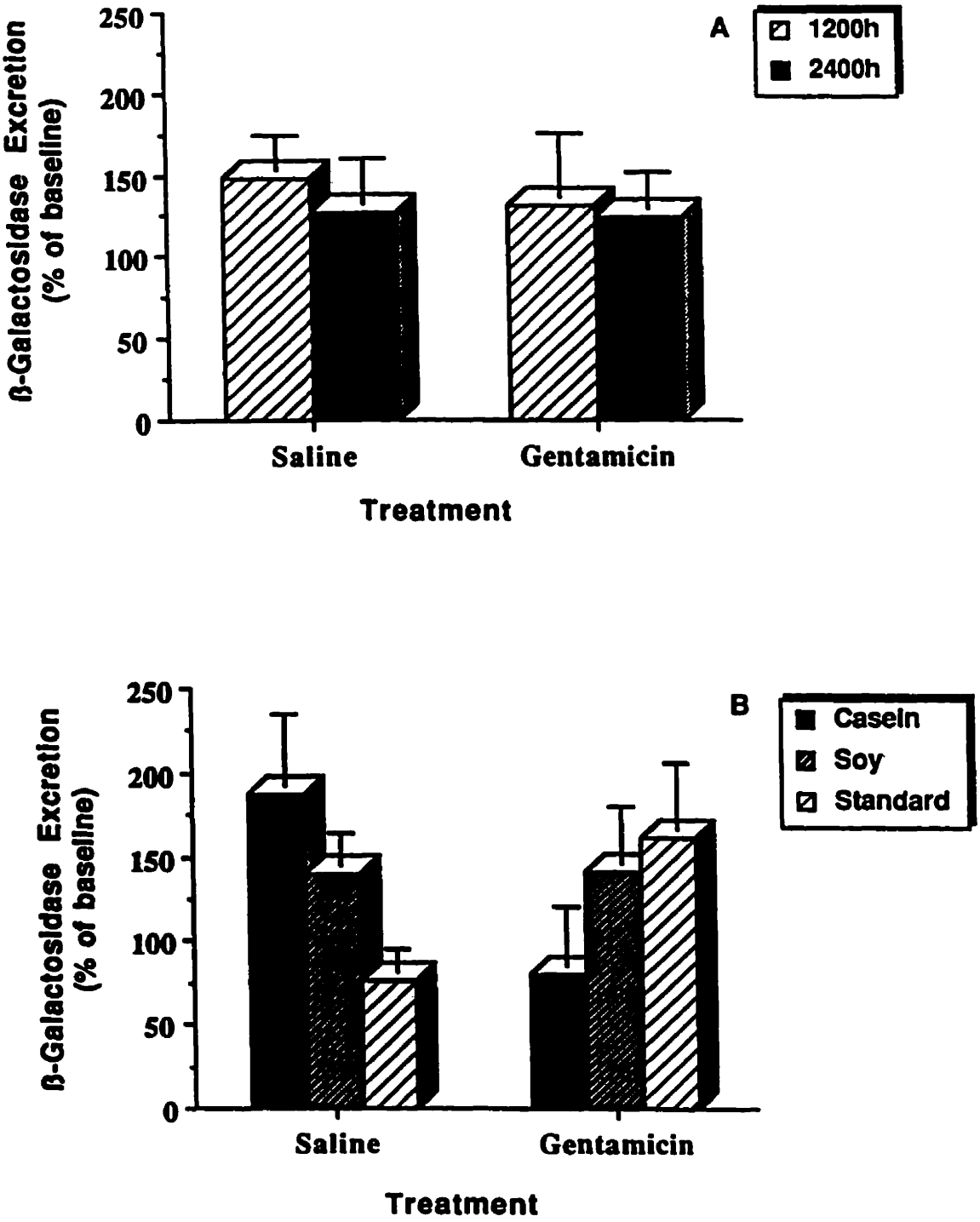


FIG. A1k: The effect of time of injection by treatment (A) and of treatment by diet (B) on β-galactosidase excretion measured ion percent of baseline in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A2 for significance.

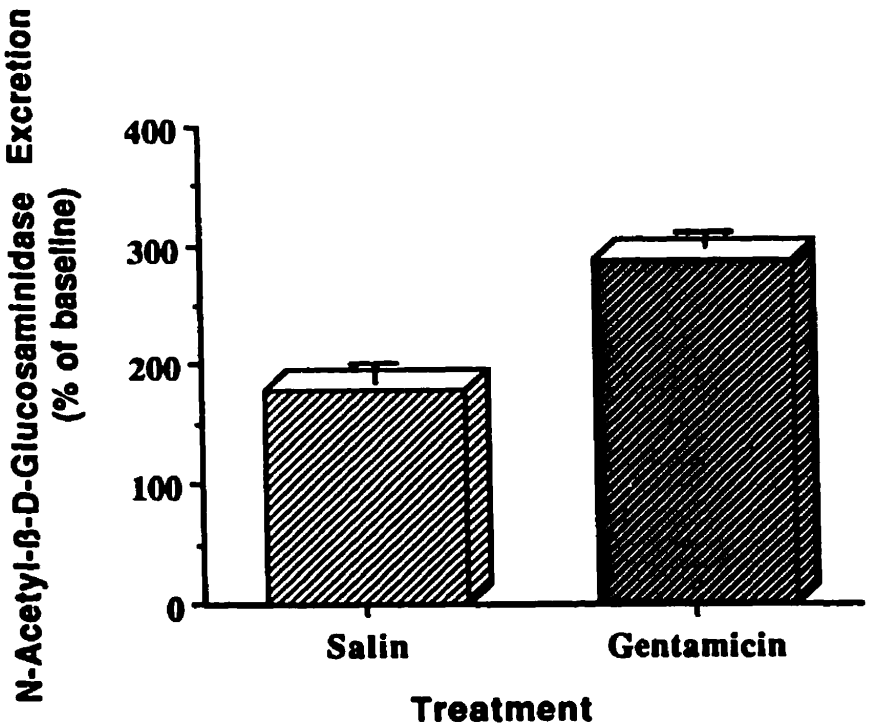


FIG. A1l: The effect of treatment on N-acetyl-β-D-glucosaminidase excretion measured in percent of baseline in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A1 for significance.

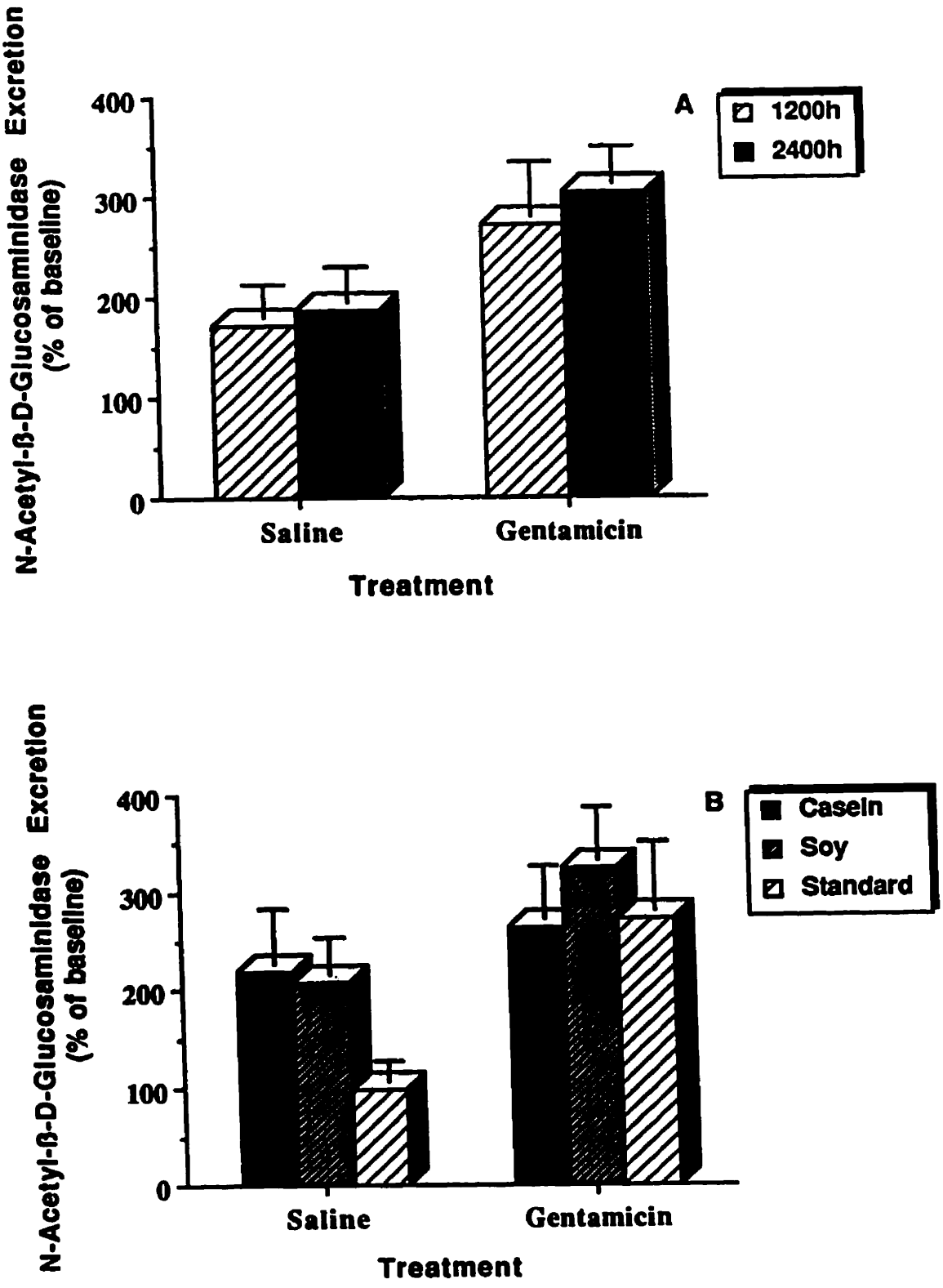


FIG. A1m: The effect of time of injection by treatment (A) and of treatment by diet (B) on N-acetyl-β-D-glucosaminidase excretion measured in percent of baseline in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A2 for significance.

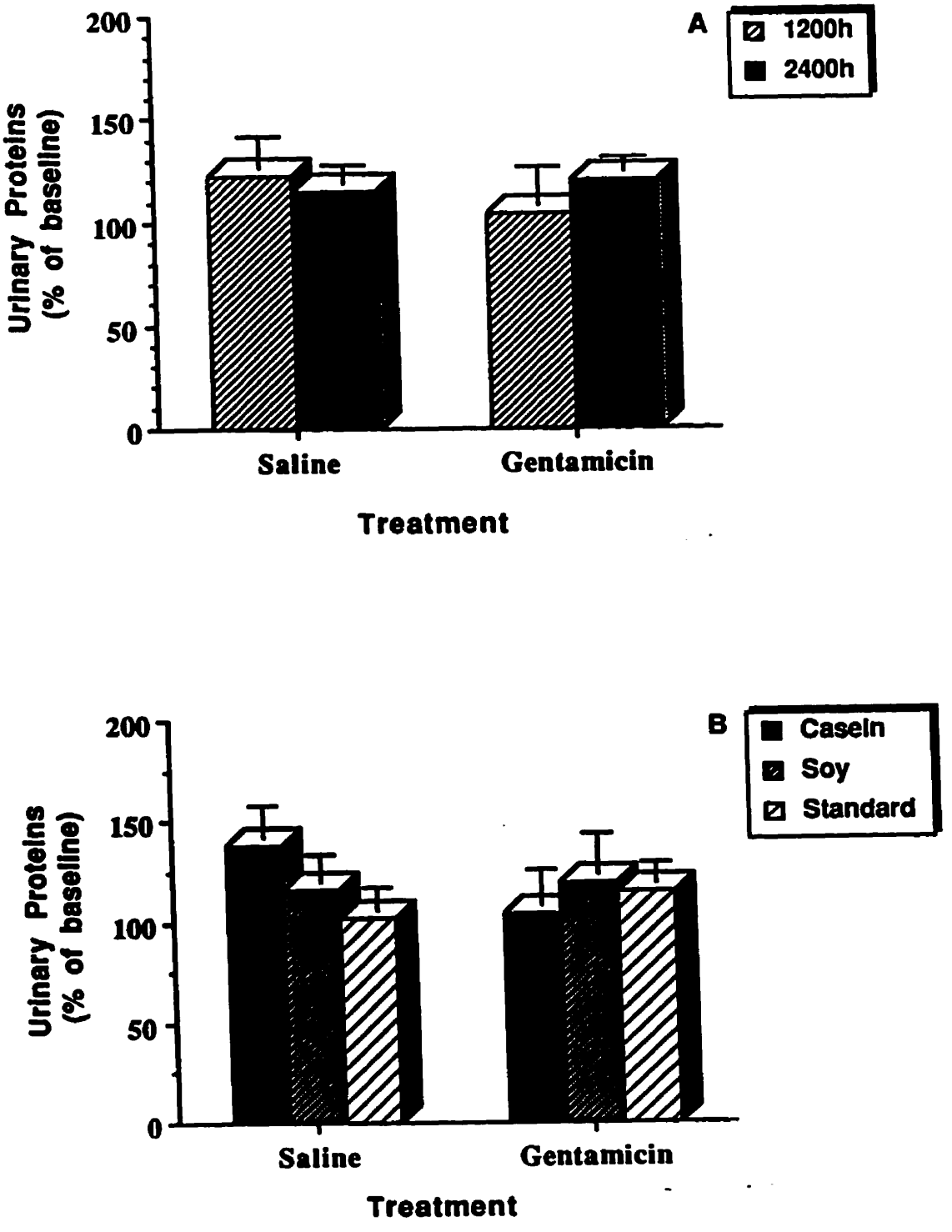


FIG. A1n: The effect of time of injection by treatment (A) and of treatment by diet (B) on urinary proteins measured in percent of baseline in rats fed a casein-, a soy-containing or a standard chow diet injected with saline or gentamicin (40 mg/kg, i.p.) at 1200 h or at 2400 h. Data expressed as mean ± SEM. See Table A2 for significance.

TABLE A3. Modified repeated measures ANOVA[1] on body weight (g) of rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

	TIME OF INJECTION						
	1200 H			2400 H			
	Test	F value	Probability	Test	F value	Probability	
MAIN EFFECTS							
TREATMENT		(1,42)=8.26	0.0063		(1,42)=0.05	0.8263	
DIET		(2,42)=2.80	0.0724		(2,42)=2.42	0.1014	
TREATMENT *DIET		(2,42)=2.11	0.1345		(2,42)=2.45	0.0989	
BETWEEN SUBJECTS INTERACTIONS							
DAY	G-G	(14,588)=24.75	0.0001	G-G	(14,588)=41.10	0.0001	
	H-F		0.0001	H-F		0.0001	
DAY*TREATMENT	G-G	(14,588)=53.07	0.0001	G-G	(14,588)=5.49	0.0005	
	H-F		0.0001	H-F		0.0001	
DAY*DIET	G-G	(28,588)=1.88	0.0685	G-G	(28,588)=1.51	0.1611	
	H-F		0.0519	H-F		0.1425	
DAY*TREATMENT*DIET	G-G	(28,588)=8.34	0.0001	G-G	(28,588)=1.93	0.0642	
	H-F		0.0001	H-F		0.0484	
EPSILON	G-G	0.2751		G-G	0.2668		
	H-F	0.3424		H-F	0.3310		

[1] The within subject effect (i.e., day main effect) is tested in the modified ANOVA. In this testing procedure, the probabilities of signifiance P>F are ajusted 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 consider 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.

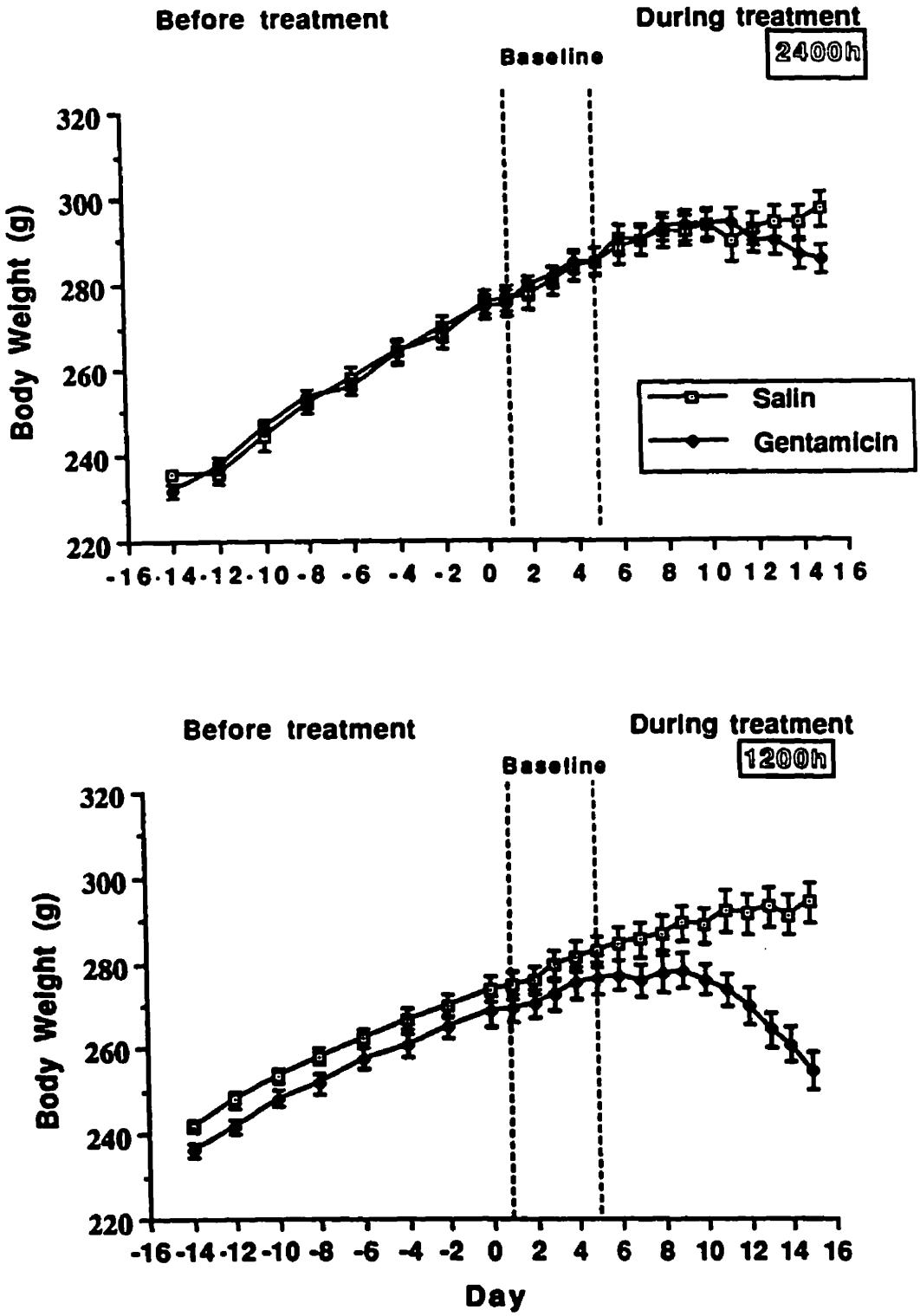
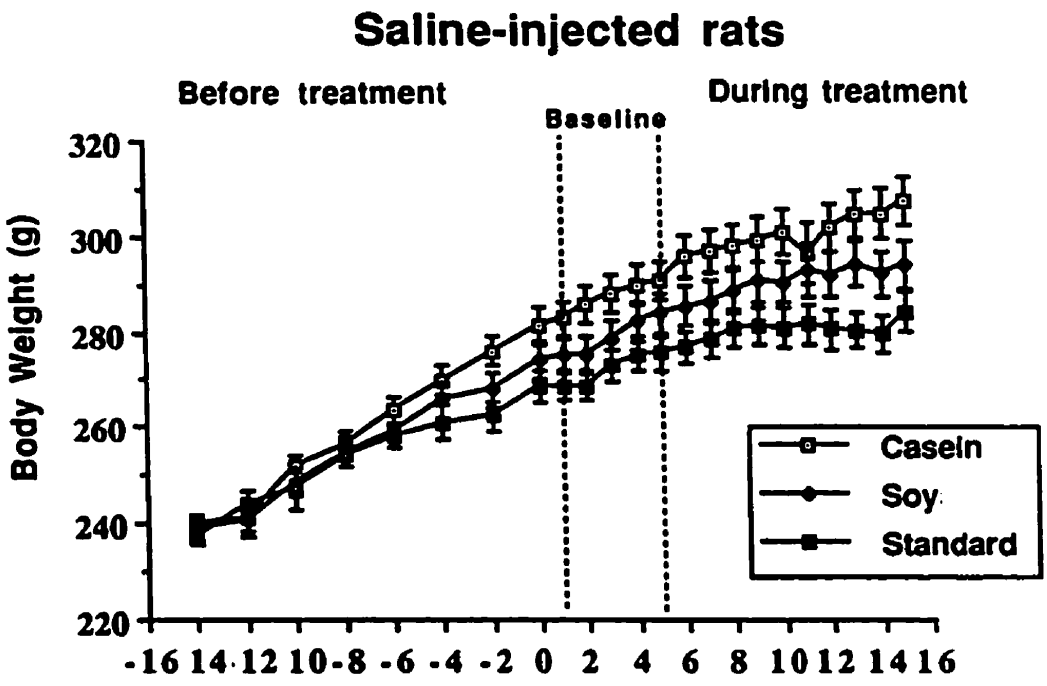


FIG. A2a: Body weights of rats throughout the experimental period where rats were fed a casein-, a soy-containing or a standard chow diet. Days –14 to 0 correspond to the adaptation period, days 1 to 5 correspond to the pretreatment (baseline) period, and days 6 to 15 correspond to the treatment period during which rats were treated with saline or gentamicin (40 mg/kg, i.p.) at 2400 h (A) or 1200 h (B). Data expressed as mean ± SEM. See Table A3 for significance.

A



B

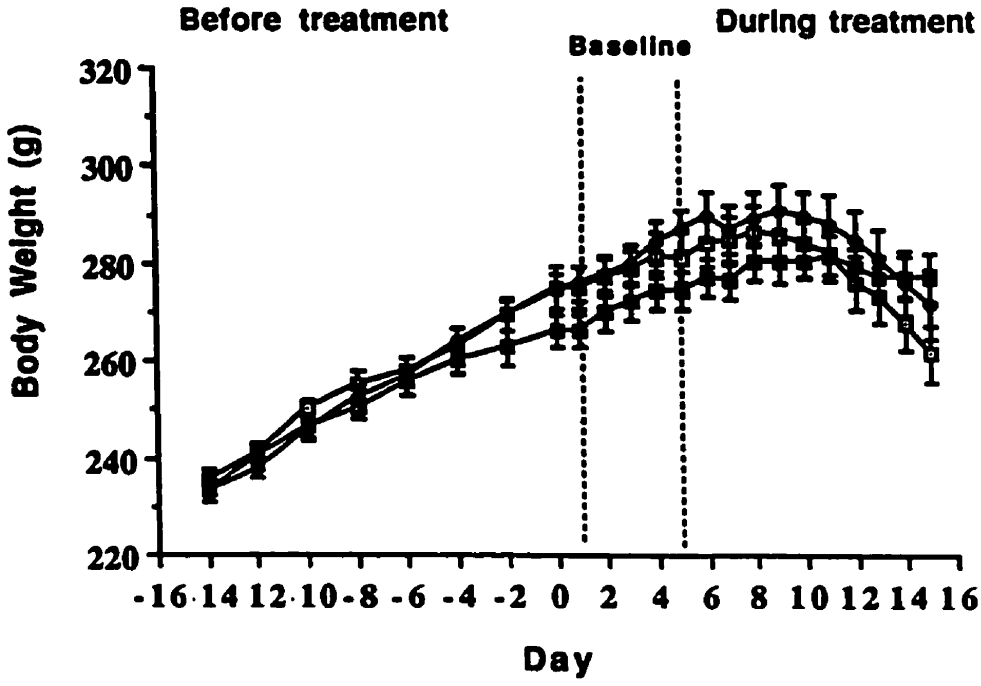
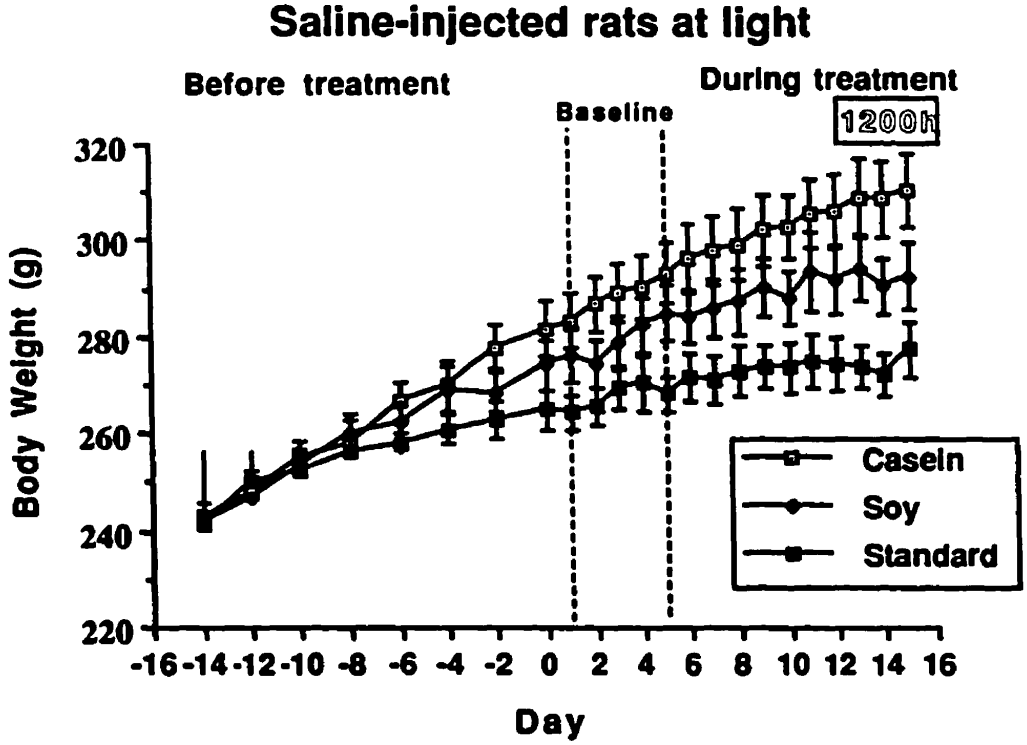


FIG. A2b: Body weights of rats throughout the experimental period where rats were fed a casein-, a soy-containing or a standard chow diet. Days −14 to 0 correspond to the adaptation period, days 1 to 5 correspond to the pretreatment (baseline) period, and days 6 to 15 correspond to the treatment period where rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 1200 h or 2400 h. Data expressed as mean ± SEM. See Table A3 for significance.



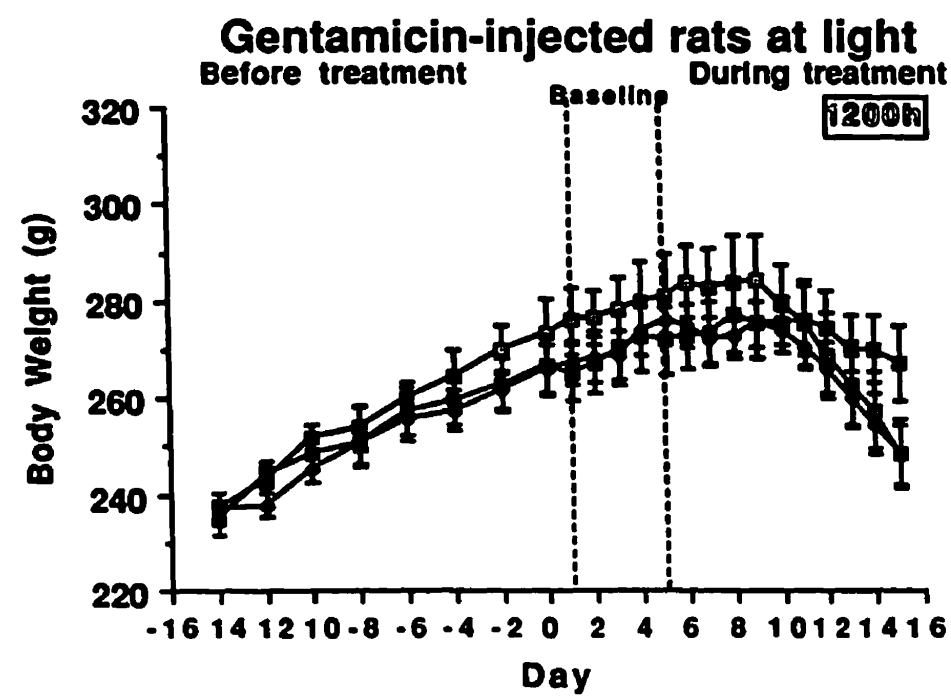
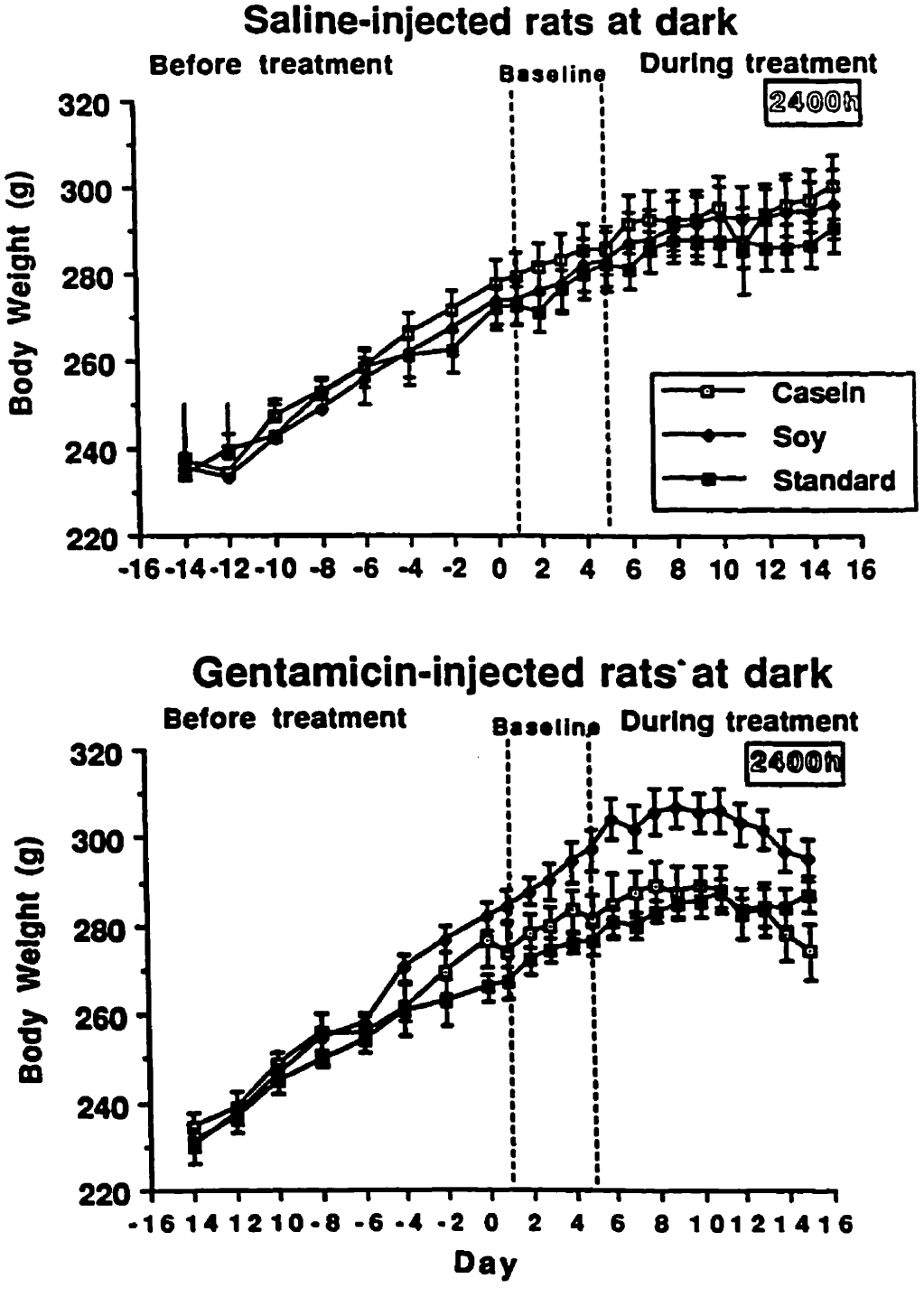


FIG. A2c: Body weights of rats throughout the experimental period where rats were fed a casein-, a soy-containing or a standard chow diet. Days −14 to 0 correspond to the adaptation period, days 1 to 5 correspond to the pretreatment (baseline) period, and days 6 to 15 correspond to the treatment period where rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 1200 h. Data expressed as mean ± SEM. See Table A3 for significance.



B

FIG. A2d: Body weights of rats throughout the experimental period where rats were fed a casein-, a soy-containing or a standard chow diet. Days –14 to 0 correspond to the adaptation period, days 1 to 5 correspond to the pretreatment (baseline) period, and days 6 to 15 correspond to the treatment period where rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 2400 h. Data expressed as mean ± SEM. See Table A3 for significance.

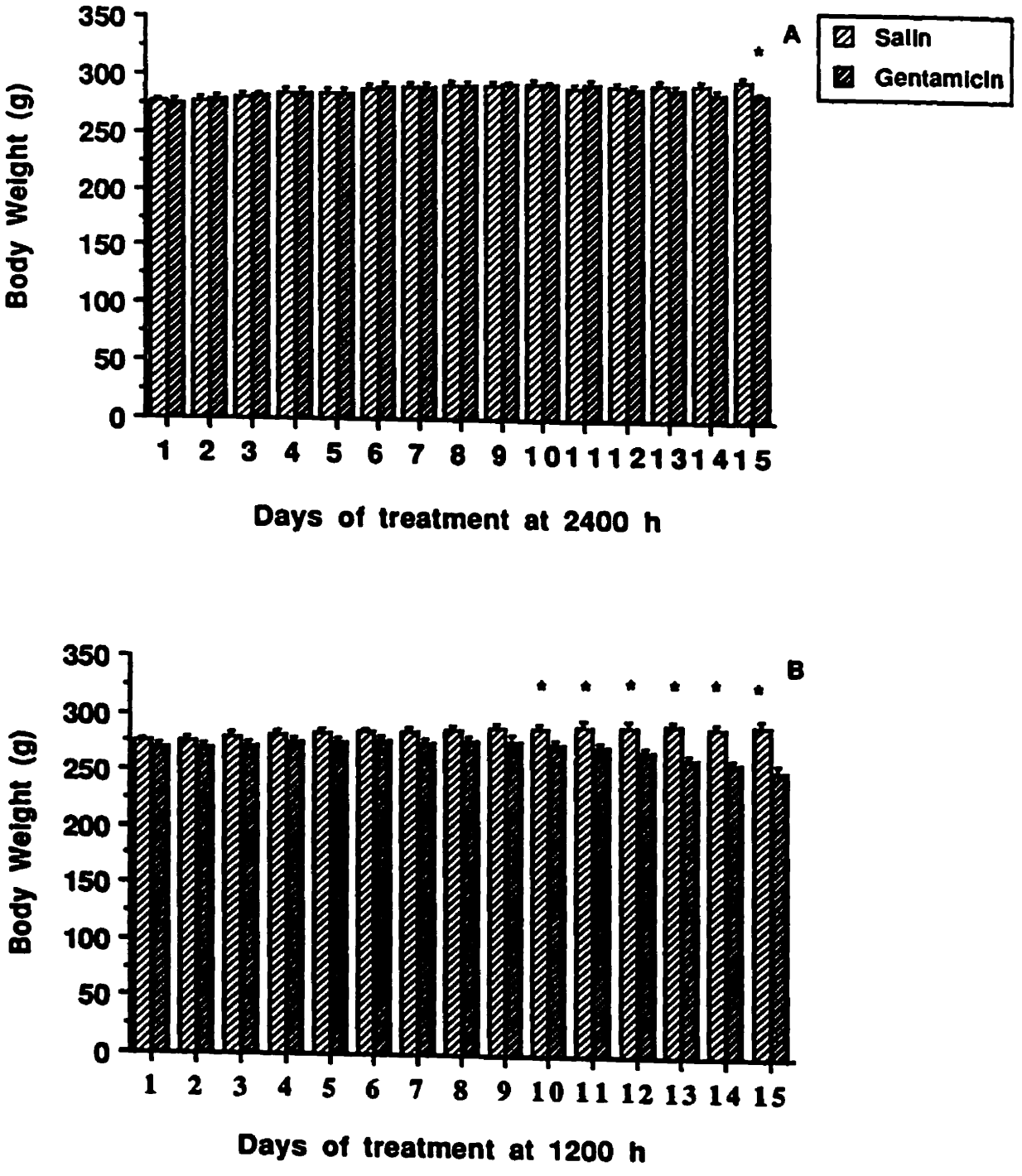


FIG. A2e: Histograms of body weights of rats throughout the experimental period where rats were fed a casein-, a soy-containing or a standard chow diet. Days –14 to 0 correspond to the adaptation period, days 1 to 5 correspond to the pretreatment (baseline) period, and days 6 to 15 correspond to the treatment period where rats were treated with saline or gentamicin (40 mg/kg, i.p.) at 1200 h (A) or at 2400 h (B). Data expressed as mean ± SEM. See Table A3 for significance.

TABLE A4. Modified repeated measures ANOVA[1] of the 24-h food intake (g) of rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

	TIME OF INJECTION						
	1200 H			2400 H			
	Test	F value	Probability	Test	F value	Probability	
MAIN EFFECTS							
TREATMENT		(1,42)=58.00	0.0001		(1,42)=24.61	0.0001	
DIET		(2,42)=13.80	0.0001		(2,42)=41.93	0.0001	
TREATMENT*DIET		(2,42)=5.88	0.0056		(2,42)=1.53	0.2279	
BETWEEN SUBJECTS INTERACTIONS							
DAY	G-G	(14,588)=28.37	0.0001	G-G	(14,588)=26.74	0.0001	
	H-F		0.0001	H-F		0.0001	
DAY*TREATMENT	G-G	(14,588)=13.79	0.0001	G-G	(14,588)=5.88	0.0001	
	H-F		0.0001	H-F		0.0001	
DAY*DIET	G-G	(28,588)=2.91	0.0006	G-G	(28,588)=1.78	0.0420	
	H-F		0.0001	H-F		0.0238	
DAY*TREATMENT*DIET	G-G	(28,588)=2.04	0.0185	G-G	(28,588)=2.19	0.0085	
	H-F		0.0086	H-F		0.0030	
EPSILON	G-G	0.4608		G-G	0.4926		
	H-F	0.6182		H-F	0.6695		

[1] The within subject effect (i.e., day main effect) is tested in the modified ANOVA. In this testing procedure, the probabilities of signifiance P>F are ajusted 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 consider 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.

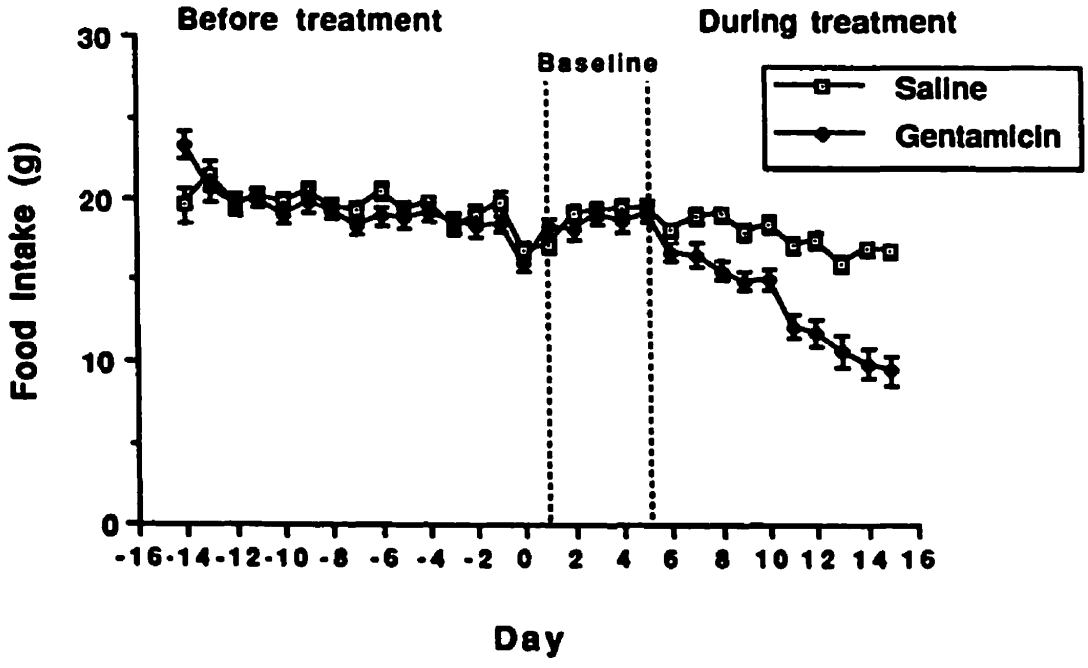


FIG. A3a: The effect of day by treatment on food intake over 24-h throughout the experimental period. Days –15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A4 for significance.

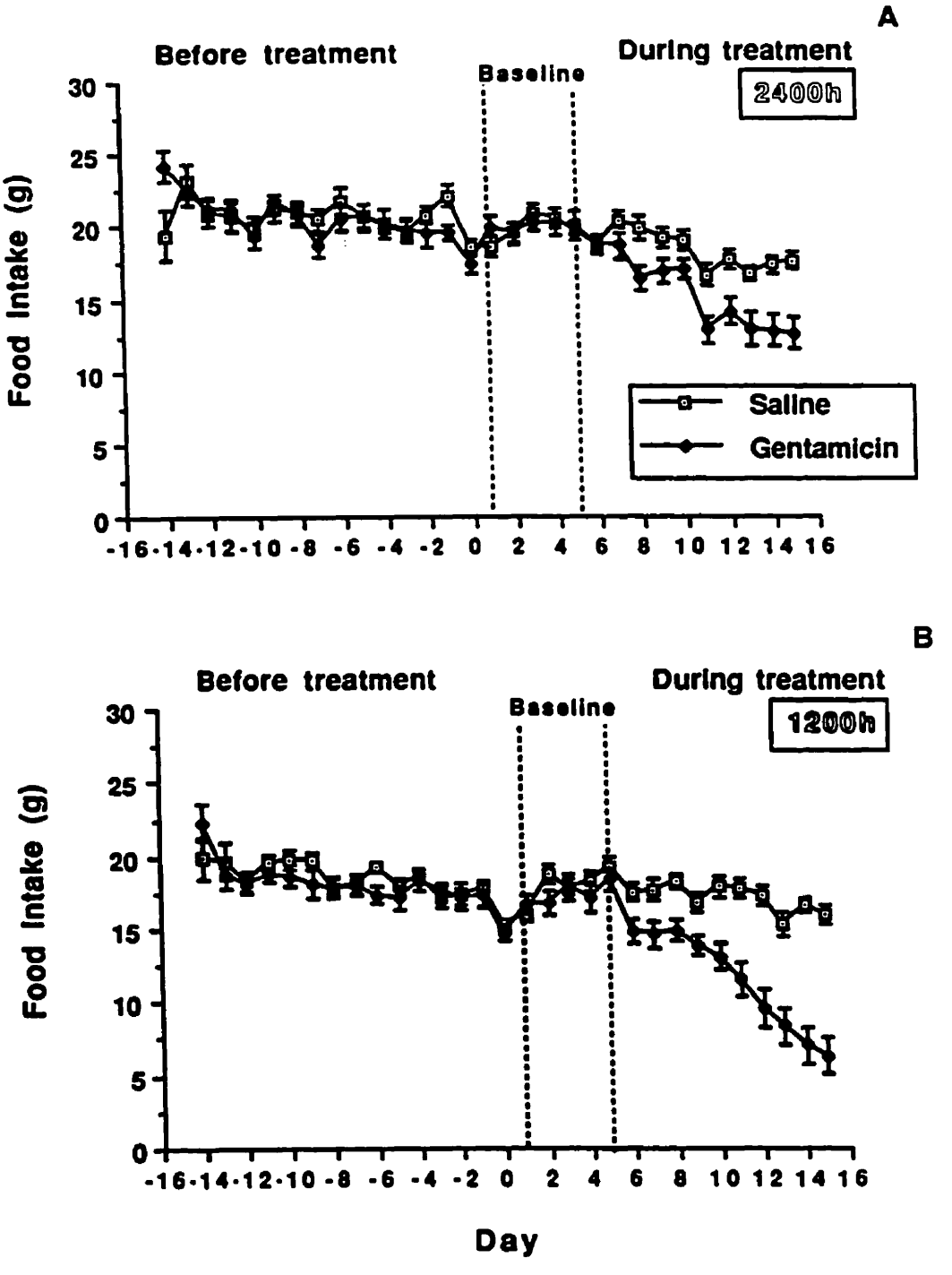


FIG. A3b: The effect of day by time of injection by treatment on food intake over 24-h throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated at 2400 h (A) or 1200 h (B) with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A4 for significance.

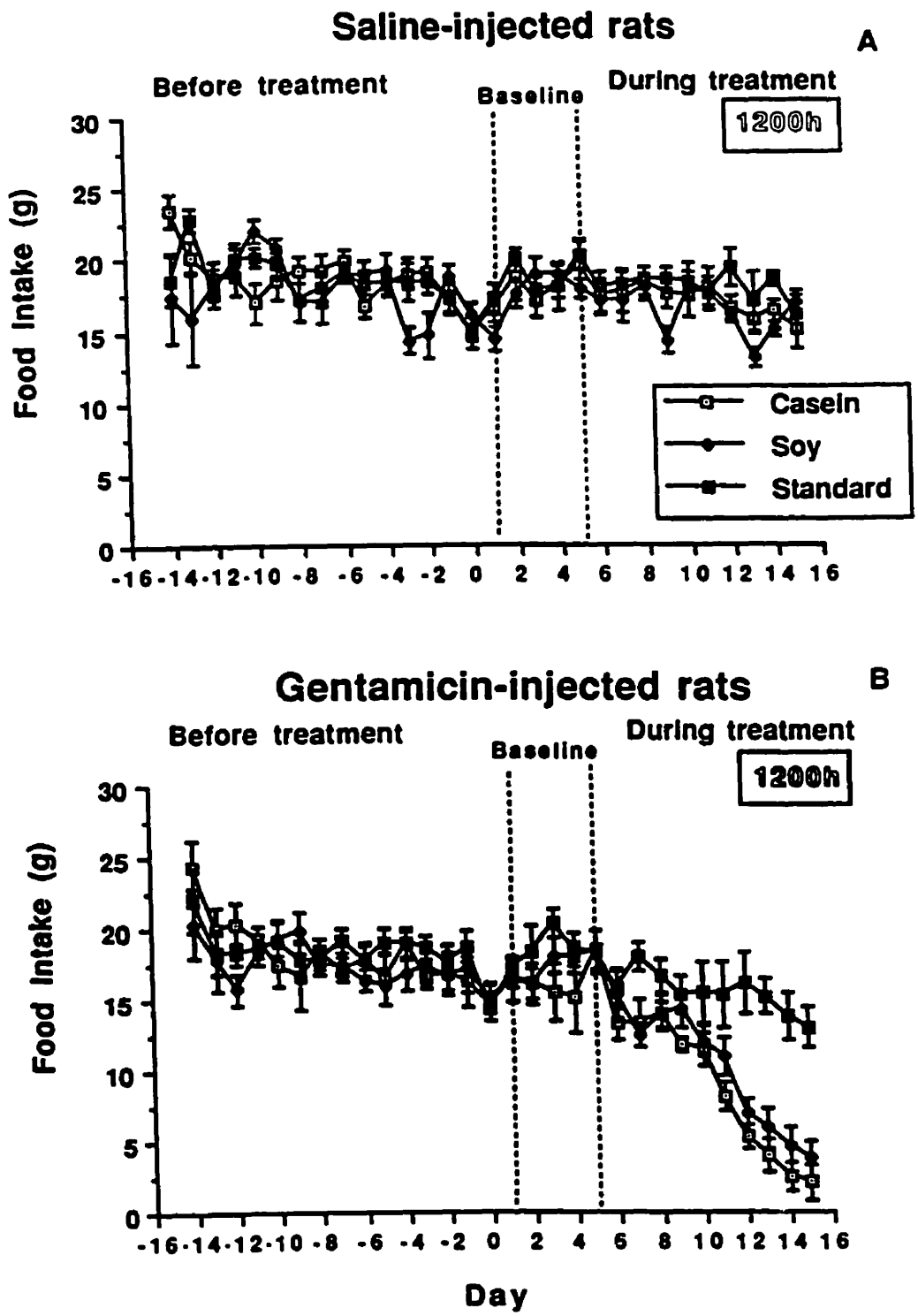


FIG. A3c: The effect of day by time of injection by diet on food intake over 24-h throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 1200 h. Data expressed as mean ± SEM. See Table A4 for significance.

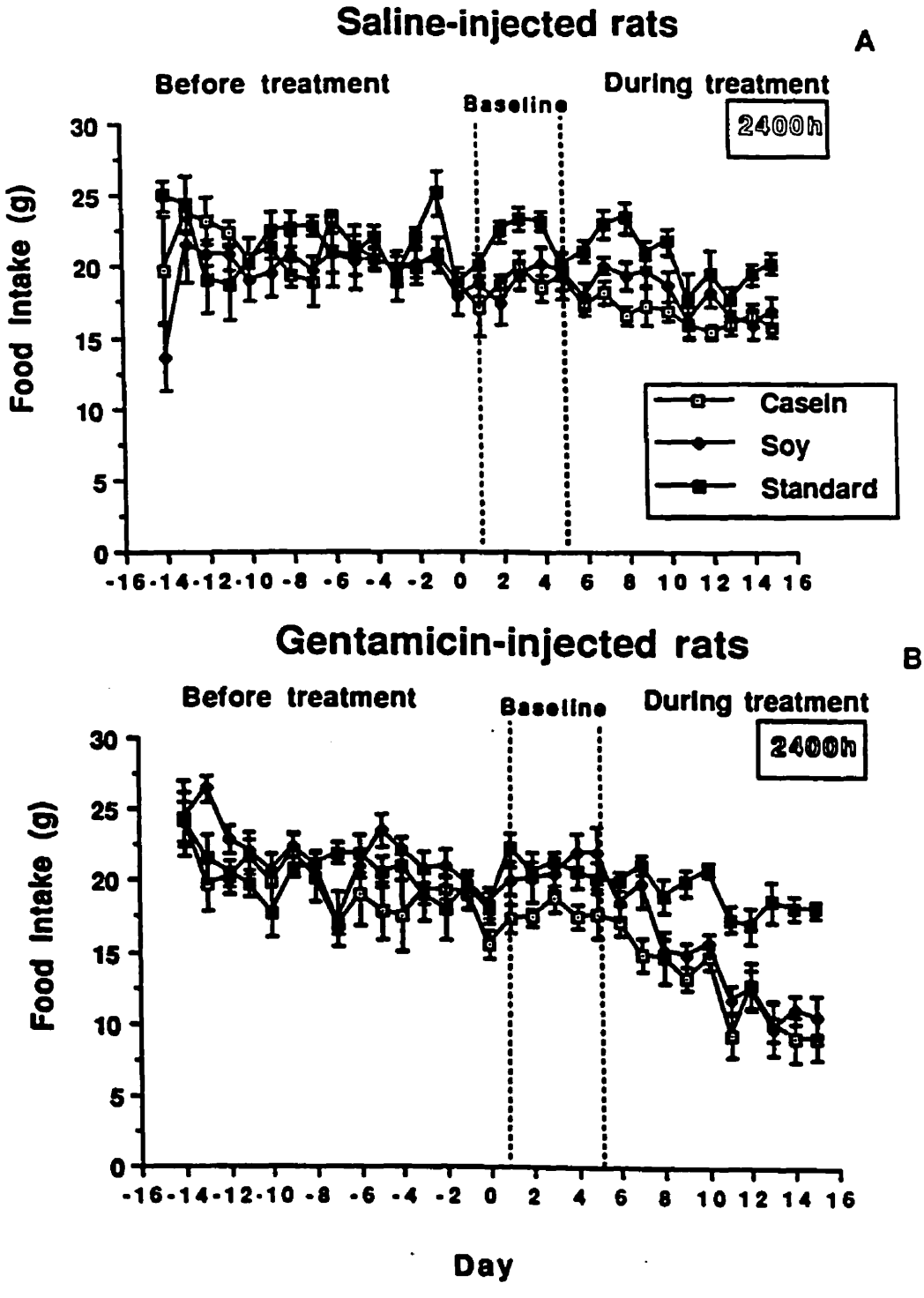


FIG. A3d: The effect of day by time of injection by diet on food intake over 24-h throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 2400 h. Data expressed as mean ± SEM. See Table A4 for significance.

TABLE A5. Modified repeated measures ANOVA[1] of the 12-h food intake (g) measured during the light period (0600 h to 1800 h) of rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

	TIME OF INJECTION						
	1200 H			2400 H			
	Test	F value	Probability		Test	F value	Probability
MAIN EFFECTS							
TREATMENT		(1,42)=30.87	0.0001			(1,42)=5.97	0.0189
DIET		(2,42)=9.57	0.0004			(2,42)=11.06	0.0001
TREATMENT*DIET		(2,42)=2.18	0.1259			(2,42)=0.06	0.9411
BETWEEN SUBJECTS INTERACTIONS							
DAY	G-G	(14,588)=10.01	0.0001		G-G	(14,588)=2.75	0.0053
	H-F		0.0001		H-F		0.0014
DAY*TREATMENT	G-G	(14,588)=7.58	0.0001		G-G	(14,588)=5.04	0.0001
	H-F		0.0001		H-F		0.0001
DAY*DIET	G-G	(28,588)=2.83	0.0011		G-G	(28,588)=2.73	0.0003
	H-F		0.0002		H-F		0.0001
DAY*TREATMENT*DIET	G-G	(28,588)=1.29	0.2227		G-G	(28,588)=1.76	0.0825
	H-F		0.1992		H-F		0.0154
EPSILON	G-G	0.4360			G-G	0.5984	
	H-F	0.5790			H-F	0.8411	

[1] The within subject effect (i.e., day main effect) is tested in the modified ANOVA. In this testing procedure, the probabilities of significance P>F are ajusted 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 consider 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.

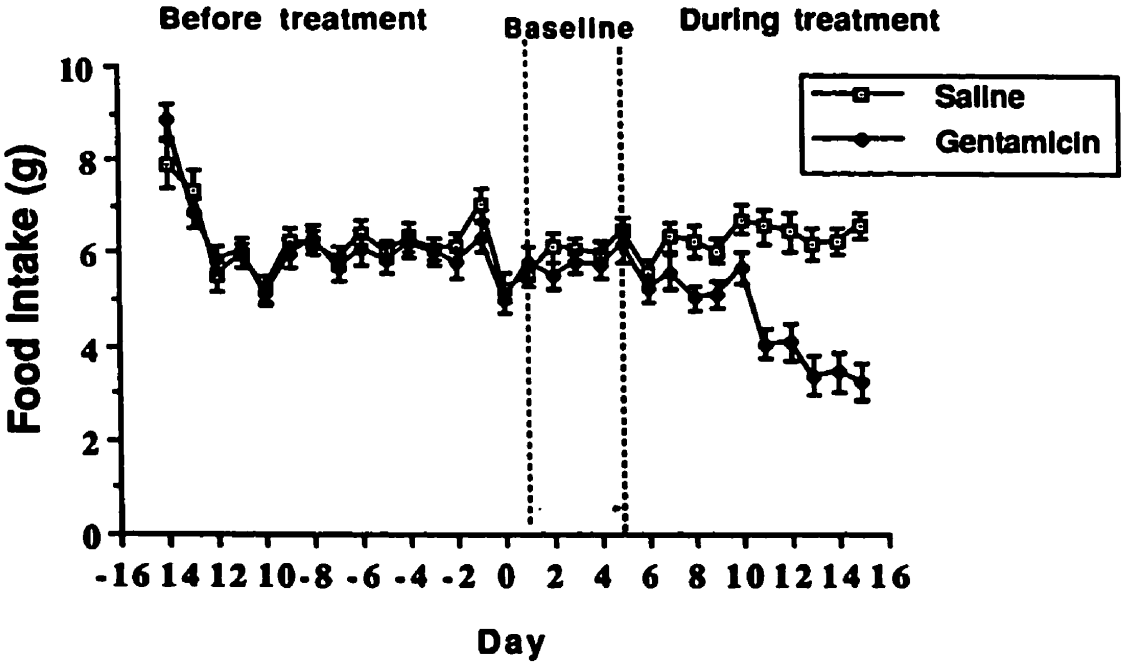


FIG. A4a: The effect of day by treatment on food intake over 12-h light throughout the experimental period. Days –15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A5 for significance.

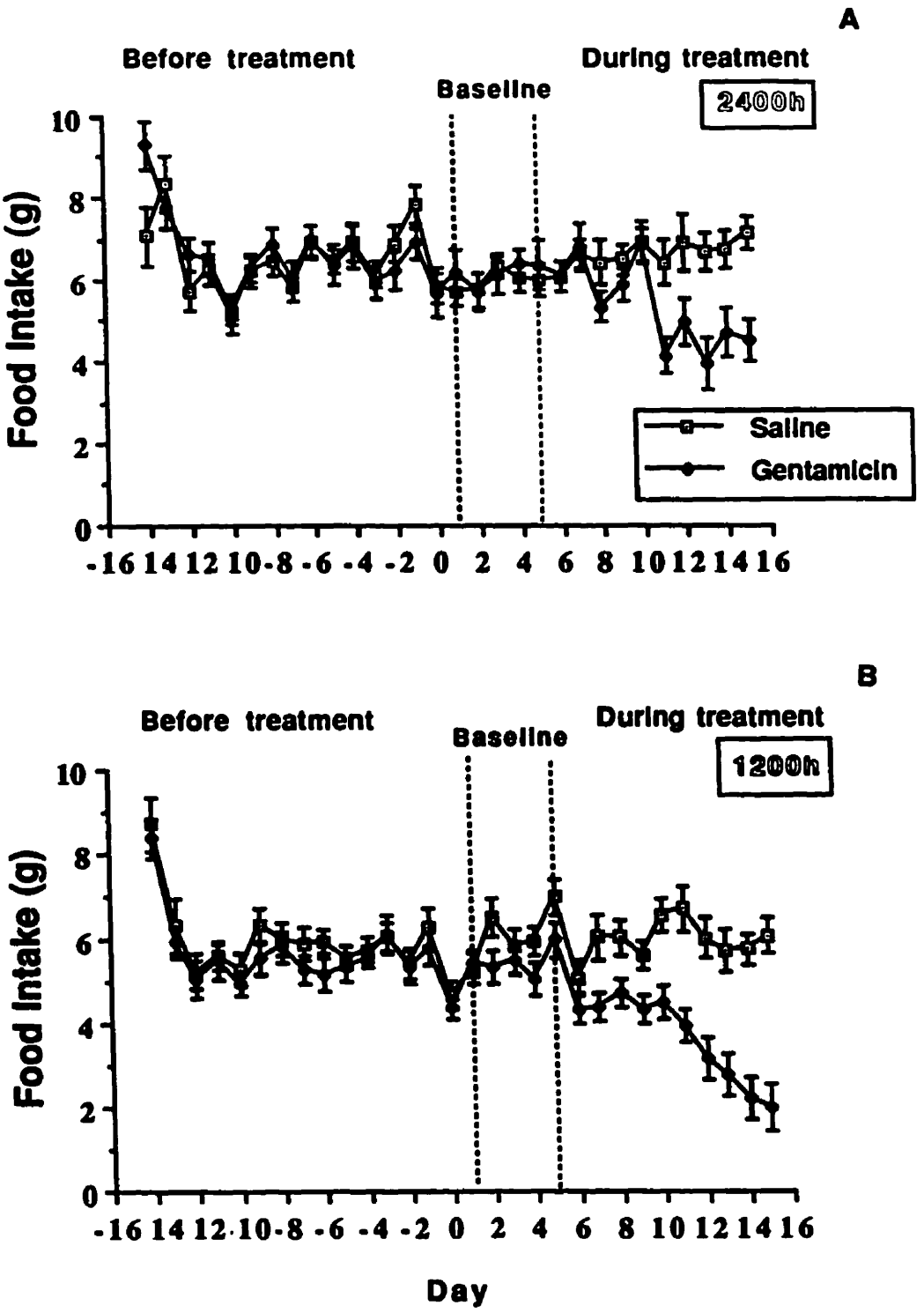


FIG. A4b: The effect of day by time of injection by treatment on food intake over 12-h light throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated at 2400 h (A) or 1200 h (B) with gentamicin (40 mg/kg, i.p.) or saline. Data expressed as mean ± SEM. See Table A5 for significance.

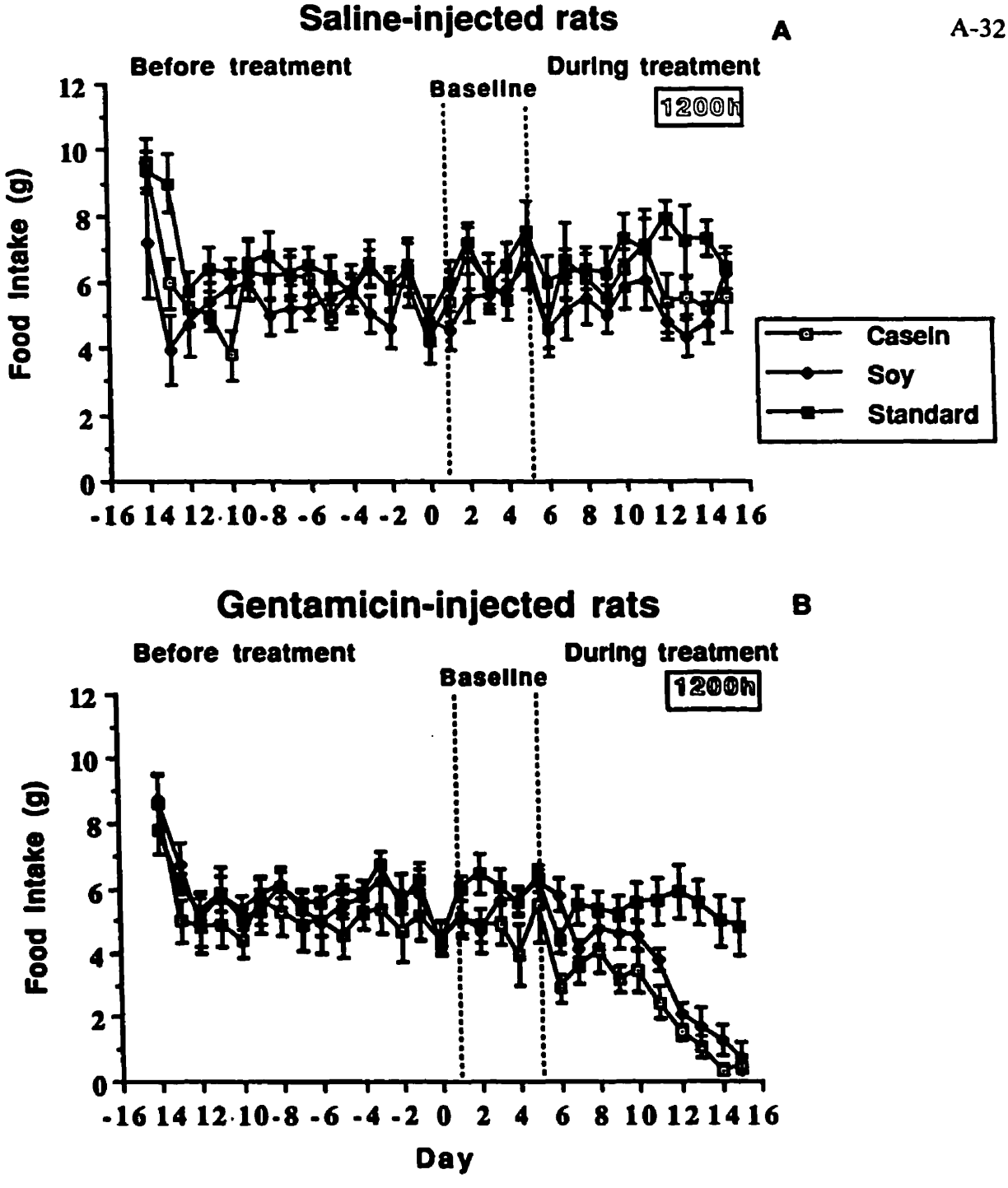


FIG. A4c: The effect of day by time of injection by diet on food intake over 12-h light throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 1200 h. Data expressed as mean ± SEM. See Table A5 for significance.

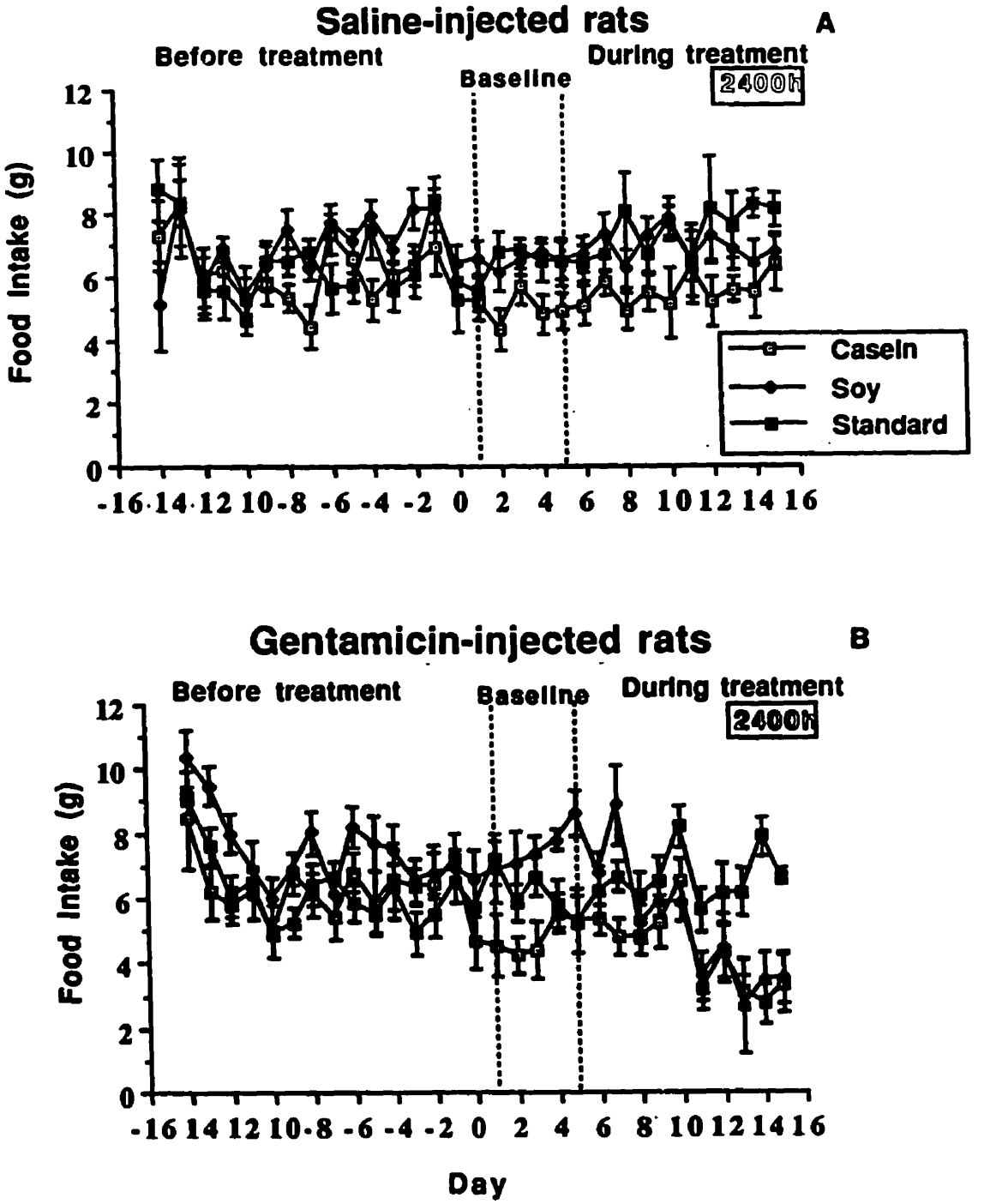


FIG. A4d: The effect of day by time of injection by diet on food intake over 12-h light throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 2400 h. Data expressed as mean ± SEM. See Table A5 for significance.

TABLE A6. Modified repeated measures ANOVA[1] of the 12-h food intake (g) measured during the dark period (1800 h to 0600 h) of rats injected at 1200 h or 2400 h with gentamicin (40 mg/kg, i.p.) or saline and fed with a casein-, a soy-containing, or a standard chow diet.

	TIME OF INJECTION							
	1200 H				2400 H			
	Test	F value	Probability		Test	F value	Probability	
MAIN EFFECTS								
TREATMENT		(1,42)=24.42	0.0001			(1,42)=11.65	0.0014	
DIET		(2,42)=4.17	0.0223			(2,42)=23.72	0.0001	
TREATMENT*DIET		(2,42)=3.73	0.0324			(2,42)=1.60	0.2143	
BETWEEN SUBJECTS INTERACTIONS								
DAY	G-G	(14,588)=28.42	0.0001		G-G	(14,588)=41.49	0.0001	
	H-F		0.0001		H-F		0.0001	
DAY*TREATMENT	G-G	(14,588)=10.64	0.0001		G-G	(14,588)=3.52	0.0009	
	H-F		0.0001		H-F		0.0001	
DAY*DIET	G-G	(28,588)=1.86	0.0827		G-G	(28,588)=1.47	0.1157	
	H-F		0.0175		H-F		0.0852	
DAY*TREATMENT*DIET	G-G	(28,588)=2.14	0.0112		G-G	(28,588)=1.79	0.0853	
	H-F		0.0044		H-F		0.0183	
EPSILON	G-G	0.4797			G-G	0.5318		
	H-F	0.6485			H-F	0.7346		

[1] The within subject effect (i.e., day main effect) is tested in the modified ANOVA. In this testing procedure, the probabilities of significance P>F are ajusted 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 consider 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.

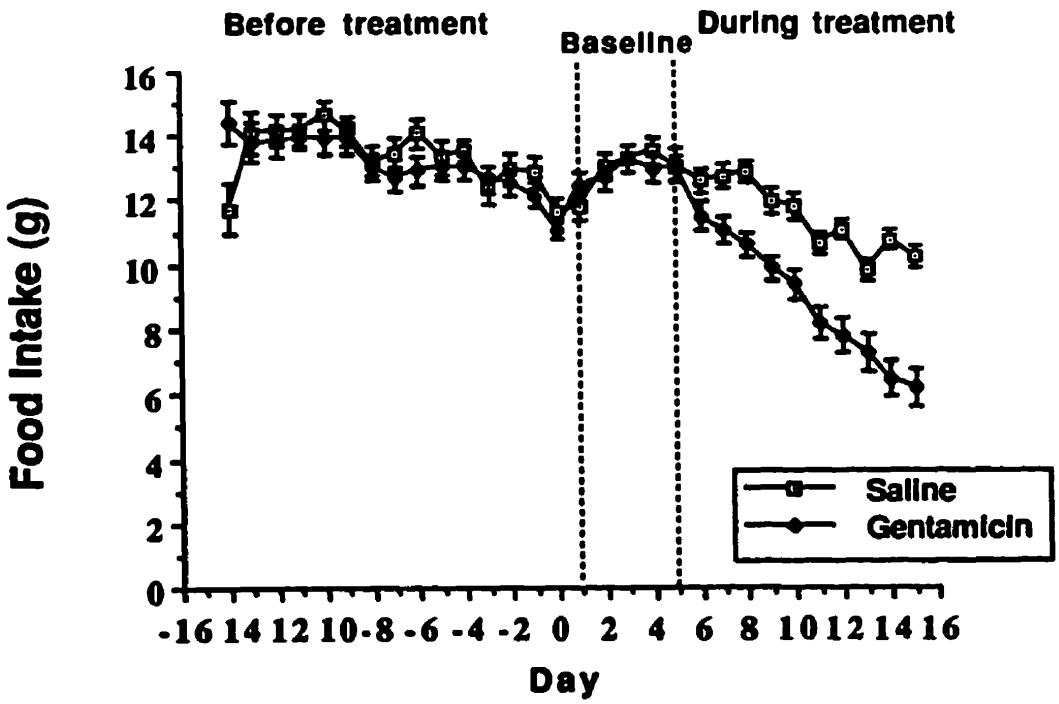


FIG. A5a: The effect of day by treatment on food intake over 12-h night throughout the experimental period. Days –15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A6 for significance.

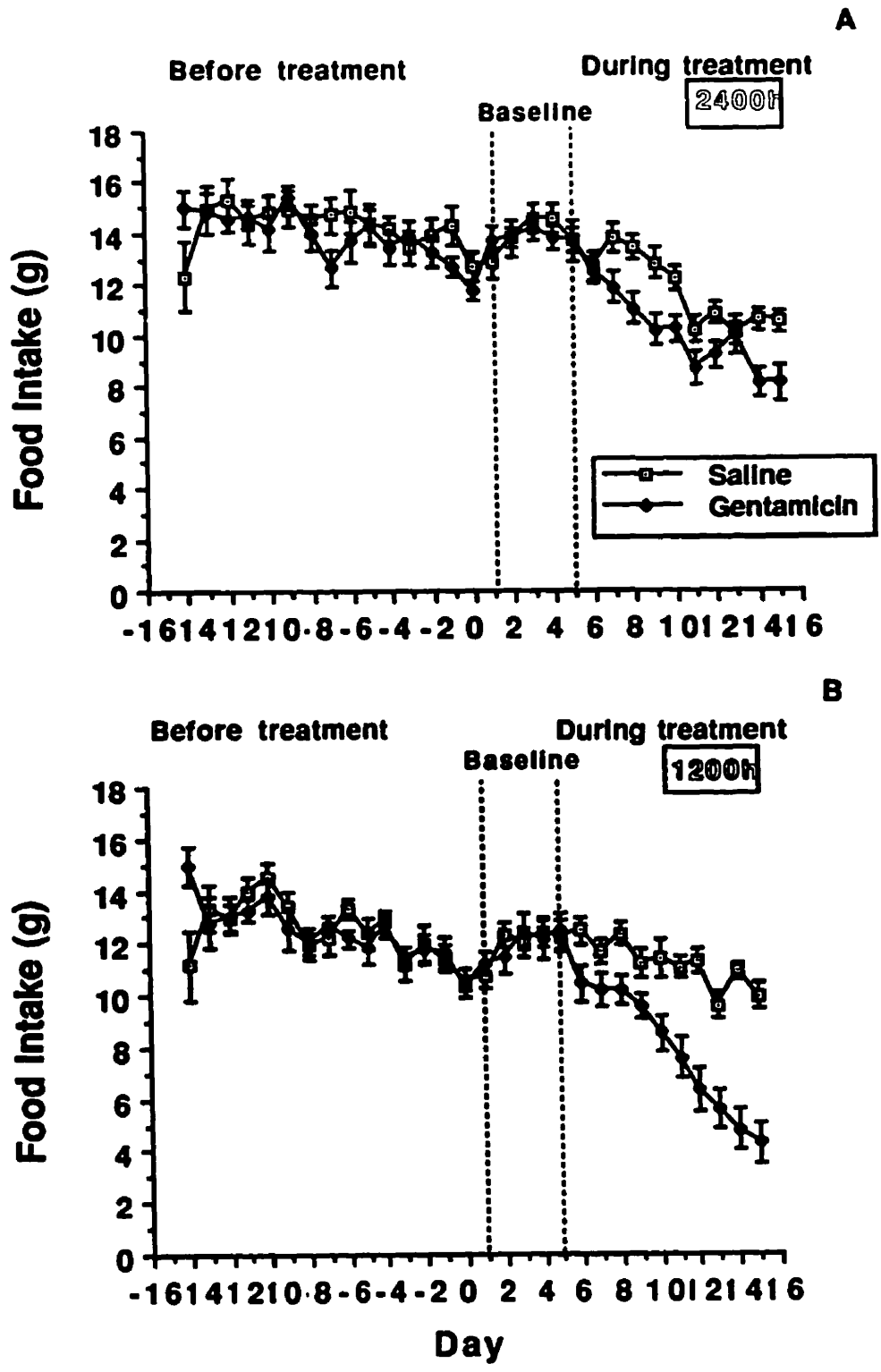


FIG. A5b: The effect of day by time of injection by treatment on food intake over 12-h night throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated at 2400 h (A) or 1200 h (B) with saline or gentamicin (40 mg/kg, i.p.). Data expressed as mean ± SEM. See Table A6 for significance.

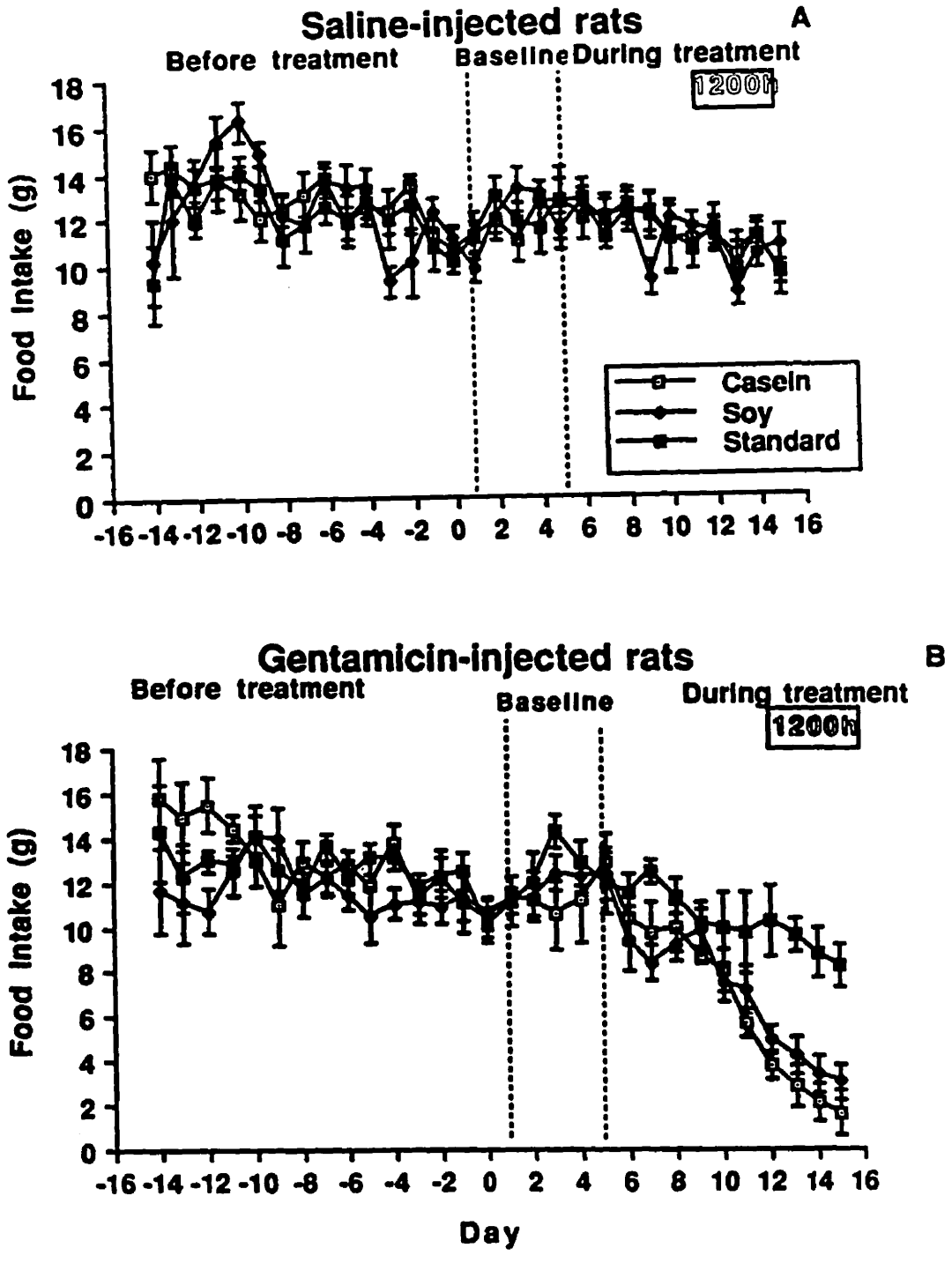


FIG. A5c: The effect of day by time of injection by diet on food intake over 12-h night throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) (B) at 1200 h. Data expressed as mean ± SEM. See Table A6 for significance.

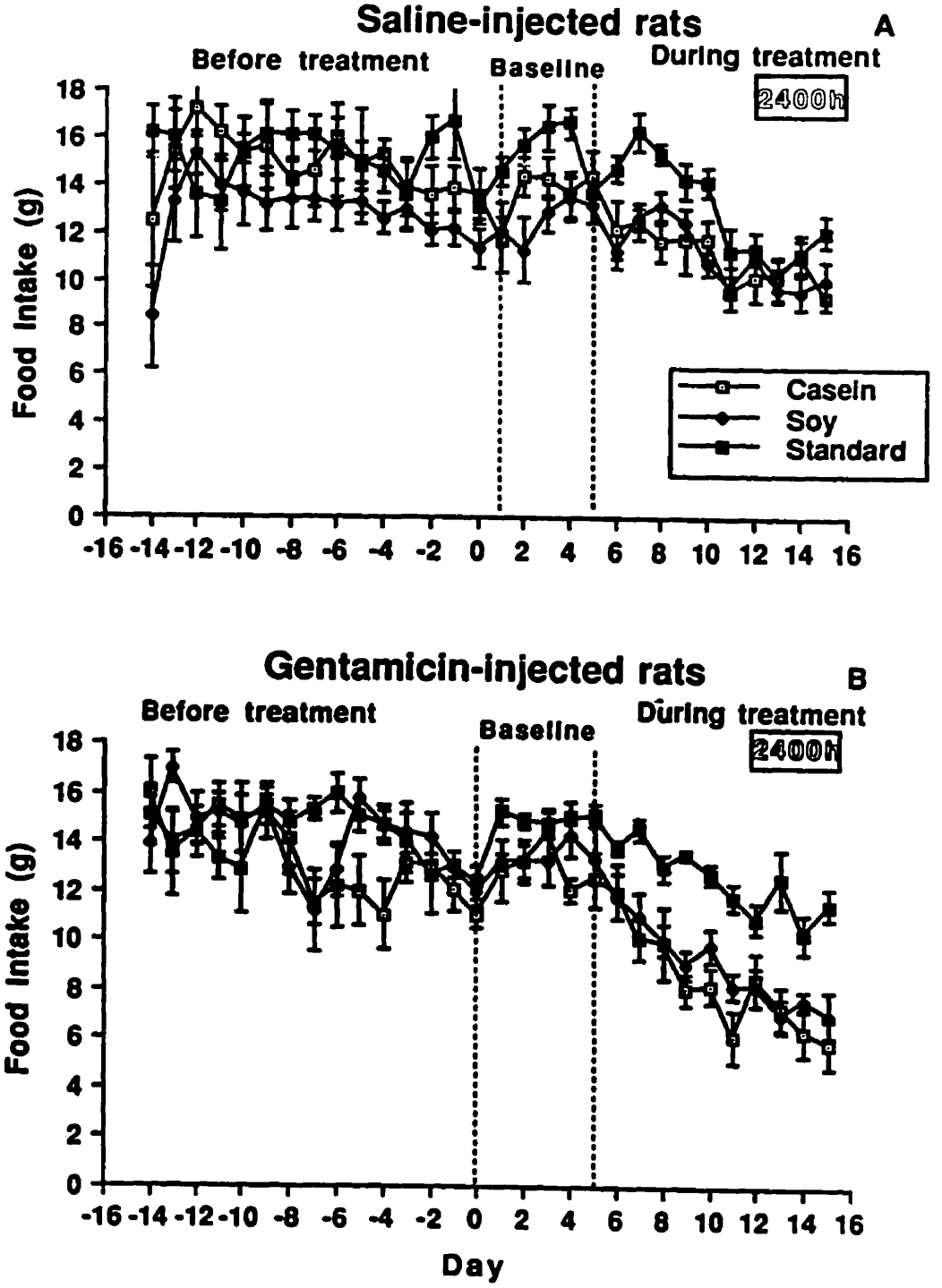


FIG. A5d: The effect of day by time of injection by diet on food intake over 12-h night throughout the experimental period. Days −15 to 0 correspond to the pretreatment period, Days 1 to 5 correspond to the baseline period and Days 6 to 10 correspond to the treatment period during which rats were treated with saline (A) or gentamicin (40 mg/kg, i.p.) at 2400 h. Data expressed as mean ± SEM. See Table A6 for significance.

Table 4a. Experimental protocol for dark period*

Period	Day	Time	Treatment
Adaptation	1 to 14	18h00	12h light F.I. measurement
		06h00	12h dark F.I. measurement
			B.wt. measurement q 2 days
Metabolic cage	15		Initial urine data (24h)
Baseline	16 to 20	18h00	12h light F.I. measurement
		06h00	12h dark F.I. measurement
			B.wt. measurement q day
Treatment	21 to 30	18h00	12h light F.I. measurement
		24h00	Gentamicin injection
		06h00	12h dark F.I. measurement
Metabolic cage	26 to 31		Feces/Urine collection (24h)
			Final urine data (24h)
Sacrifice	32		

Table 4b. Experimental protocol for light period*

Period	Day	Time	Treatment
Adaptation	1 to 14	18h00	12h light F.I. measurement
		06h00	12h dark F.I. measurement
			B.wt. measurement q 2 days
Metabolic cage	15		Initial urine data (24h)
Baseline	16 to 20	18h00	12h light F.I. measurement
		06h00	12h dark F.I. measurement
			B.wt. measurement q day
Treatment	21 to 30	12h00	Gentamicin injection
		18h00	12h light F.I. measurement
		06h00	12h dark F.I. measurement
Metabolic cage	26 to 31		Feces/Urine collection (24h)
			Final urine data (24h)
Sacrifice	32		

*B.wt.: Body weight * q : every *F.I. : Food intake

FIG. B: Gentamicin-induced phospholipidosis

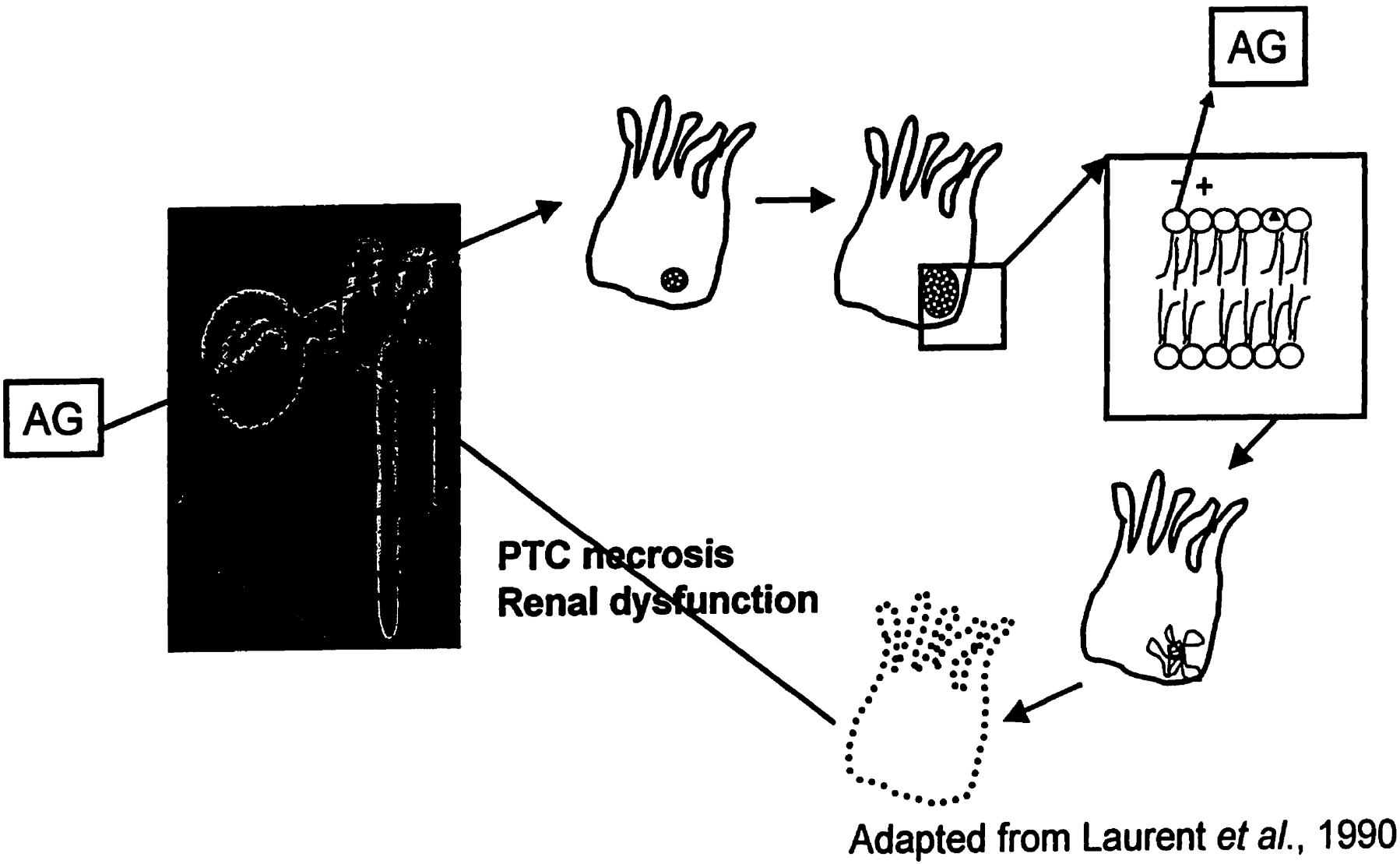
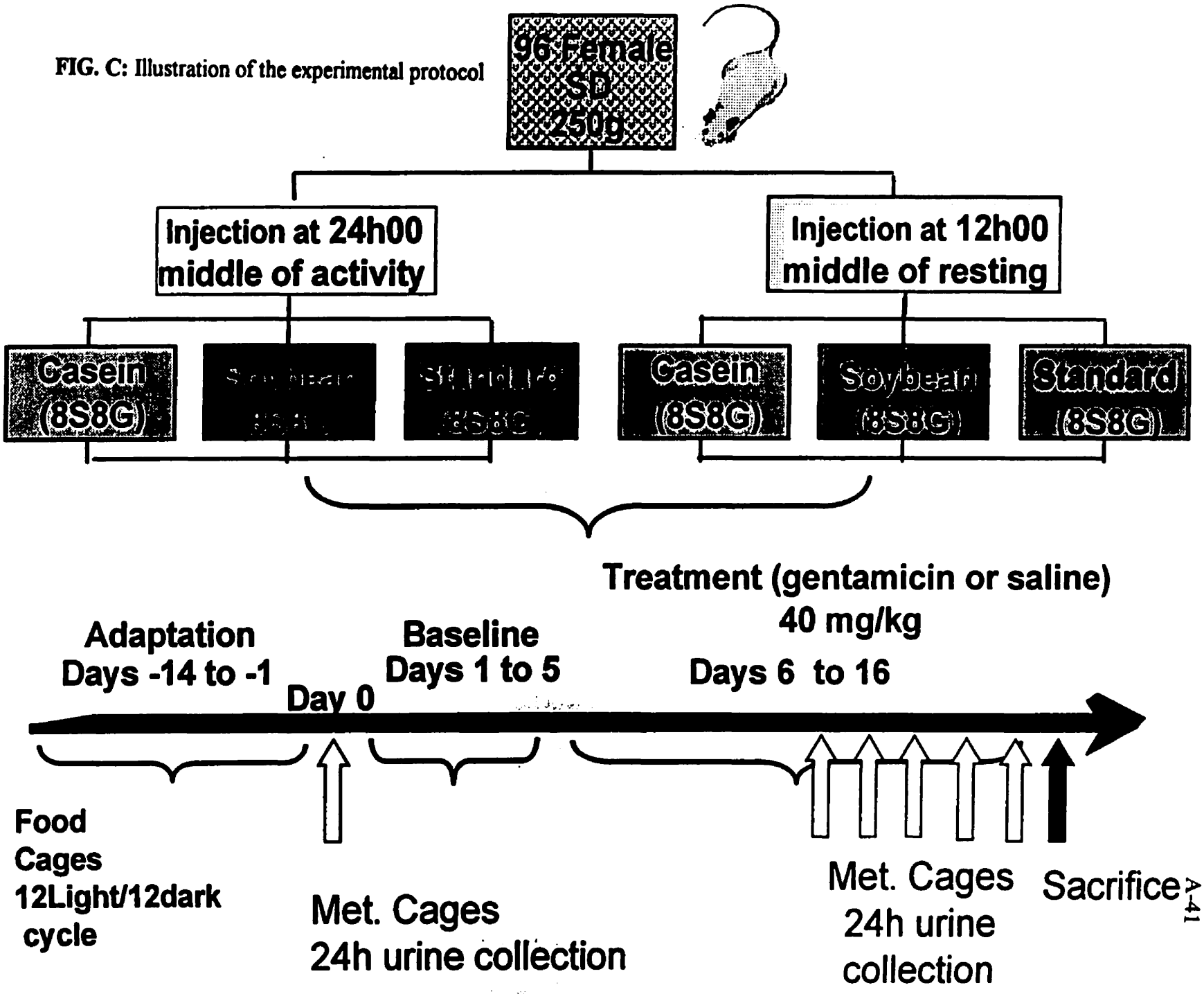


FIG. C: Illustration of the experimental protocol

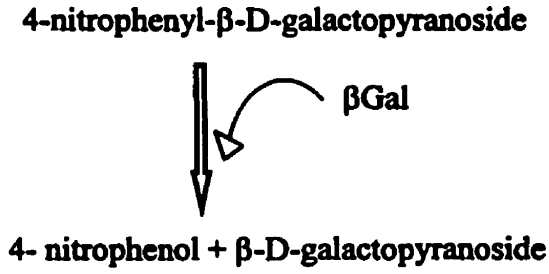


PROTOCOL FOR NEPHROTOXICITY MEASUREMENTS

1.0 Urinary enzymes activity

1.1 Urinary β-galactosidase

Principle: β-galactosidase (βGal) is found in the lysosomes of proximal tubular cells, its presence in the urine is directly proportional to nephrotoxicity. The determination of βGal activity was performed using the colorimetric method of Maruhn (1976). βGal was measured by spectrophotometric method using the absorbance of the degradation product 4-nitrophenol at 405 nm.



Material: Citric acid, anhydrous, powder

Citrate trisodium, dihydrate, granular

Para-nitrophenyl-β-D-galactopyranoside

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

Fresh 24-h urine samples

Reagents preparation:

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

-Dissolve 3.82 g of citrate anhydre 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 anhyde to citrate trisodium dihydrate.

-Conservation time: 3 months at 4°C.

B. Buffered substrate solution: PNGP 5 mM/l

-Dissolve 0.11 g of para-nitrophenyl-β-D-galactopyranoside (PNGP) in 75 mL of citrate buffer.

-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 8N

-6 mL of HCL 12N for 3 mL 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 has to corresponds to the number of urine sample 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.

-Wait 15 minutes and terminate the reaction by adding 200 µl of AMP buffer to all tubes.

-Read the content of all tubes immediately at 405 nm against the blank reagent.

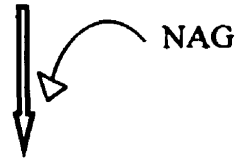
Calculation of activity:

The enzyme activity (U/l) is the difference between the tubes of buffer-substrate and citrate buffer * 10.811. The enzyme output (mM/24-h) is the enzyme activity (U/l)* diuresis (ml/24-h).

1.2 Urinary N-acetyl-β-D-glucosaminidase

Principle: N-acetyl-β-D-glucosaminidase (NAG) is found in the lysosomes of proximal tubular cells, its presence in the urine is directly proportional to nephrotoxicity. The determination of NAG activity was performed using the colorimetric method of Maruhn (1976). NAG was measured by spectrophotometric method using the absorbance of the degradation product 4-nitrophenol at 405 nm.

4-nitrophenyl-N-acetyl-β-D-glucosaminidase



4- nitrophenol + β-D-glucosaminide

Material: Citric acid, anhydrous, powder

Citrate trisodium, dihydrate, granular

Para-nitrophenyl-N-acetyl-β-D-glucosaminide

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

Fresh 24-h urine samples

Reagents preparation:

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

-Dissolve 3.82 g of citrate anhydre 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 anhyde 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-β-D-glucosaminidase (PNAG) in 100 mL of citrate buffer.

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

-Conservation time: 3 months at 4°C.

C. 2-amino-2-methyl-propan-1-ol (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 8N

-6 mL of HCL 12N for 3 mL distilled water.

Procedure:

1) Prewarm urines at 37°C in a water bath.

2) Thaw and prewarm tubes containing the buffer-substrate solution to 37°C. The number of tubes has to corresponds to the number of urine sample 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.

3) Distribute 200 µl of citrate buffer in disposible tubes (12 X 75).

4) Add 200 µl of prewarmed urines to the buffer-substrate and citrate buffer containing tubes.

5) Wait 15 minutes and terminate the reaction by adding 200 µl of AMP buffer to all tubes.

6) Read the content of all tubes immediately at 405 nm against the blank reagent.

Calculation of activity:

The enzyme activity (U/l) is the difference between the tubes of buffer-substrate and citrate buffer * 10.811. The enzyme output (mM/24-h) is the enzyme activity (U/l)* diuresis (ml/24-h).

2.0 Cortical accumulation of gentamicin

Dulbecco's phosphate-buffered saline tampon (PBS)

Material: Sodium Chloride (NaCl)
Potassium Chloride (KCl)
Potassium phosphate monobasic (KH_2PO_4)
Sodium phosphate (Na_2HPO_4)

1) Dissolve in 800 ml milliQ water:

NaCl	8.00 g
KCl	0.20 g
KH_2PO_4	1.44 g
Na_2HPO_4	3.63 g

2) Adjust pH to 7.4 with HCl or NaOH.

3) Complete the volume of the solution to 1 liter.

4) Autoclave for a sterile solution.

Homogenate preparation for microbiological dosages:

Material: Dulbecco's phosphate-buffered saline tampon solution (PBS) (2 ml/sample)
Renal cortices
Tissue-tearor RTM (Boispec Products, Bartleville, Okla.)
5 mL plastic tubes
Ice bath

1) Put 2 mL of cold PBS in 5 mL tubes.

2) Weight renal cortices (record weight), and put in the PBS tube.

3) Homogenize with a Tissue-tearor for 45 seconds or until homogeneous.

4) Freeze sample until further analysis.

Preparation of the culture medium for cortical accumulation gentamicin dosage:

Material: 2000 mL erlenmeyer
Magnetic bar
Heating plate
100 petri dishes
10 mL syringe (sterile)
40 g Tryptic Soy Agar (Difco Laboratories, Detroit, Michigan)
Capsules of *Bacillius subtilis* ATCC 6633 spores
Aluminum foil
Autoclave tape
Burner

1) Prepare a culture medium in a 2000 mL erlenmeyer by dissolving 40 g of Tryptic Soy Agar in 1 liter of milliQ water.
2) Heat almost to the boiling point with a magnetic bar.
3) Remove the magnetic bar and seal the opening with aluminum foil and autoclave tape.
4) Autoclave for 20 minutes.
5) Immediately after autoclaving, put the erlenmeyer into a 56°C water bath for one hour.
6) When the culture medium becomes lukewarm, break a capsule of *Bacillus subtilis* spores, thoroughly agitate to homogenize the culture medium.
7) Ignite a burner to create a sterile area.
8) Put 10 mL of the culture medium into 100 Petri dishes.
9) Refrigerate for 24 hours.

Microbiological dosage of gentamicin:

Material: Erlenmeyer and aspirating system
Ethanol 70%
Refrigerated culture medium in Petri dishes
Sonicator (model W-375; Bionetics Ltd., Montreal, Qc, Ca)
Vernier

1) Prepare the aspirating system by fixing a plastic tube between an open tap and an erlenmeyer and by fixing an other tube to the erlenmeyer to be used as an aspirating device.
2) Ignite a burner to create a sterile area.
3) Aspirate about 200 mL of ethanol into the erlenmeyer to disinfect the aspirating device.
4) Wait until the ethanol is evaporated on the tip of the aspirating tube.
5) Make 3 holes in the culture medium of each Petri dishes using the tip of the aspirating tube (the tube has to be placed vertically on the Petri dishes in order to make perfect circles).
6) Thaw homogenate preparation using a water bath and sonicate each sample.
7) Put 10 µl of homogenate preparation into each holes.
8) Triplicate on 3 different Petri dishes.
9) Distribute different gentamicin concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/mL to define a reference curve.
10) Triplicate also on 3 different Petri dishes.
11) Wait 24 hours.
12) Measure the diameter of the growth inhibition of *Bacillius subtilis* using a vernier.

Calculations:

-Calculate the average of the growth inhibition (mm) for each of the reference concentrations.
-Draw graph with the average of the reference concentrations (X=average diameter, Y=concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200)).
-Transpose this graph into a log scale to obtain the concentration formula for the curve.
-For each sample to be analysed, make calculations using the concentration formula to obtain (y) µg/mL:

$$y = 0.14234 * 10^{(0.13926 * x)}$$

where "x" is the average diameter for each sample

-Multiply the result of the concentration formula (µg/mL) by 2 mL (10 µl of gentamicin (40mg/mL) is equal to 2 mL of homogenate)

-Divide the previous result (µg) by the weight of the renal cortex (g) to obtain a final concentration of µg/g of renal cortex.

3.0 Corticocellular regeneration

Day 1: Preparation of required material and solutions

Day 2: DNA extraction

Day 3: DNA dosage

Day 4: Reading results and calculations

Material: Distilled water (4 °C)

1, 2, 5, and 10 mL pipettes

Automatic pipette P-5000

Centrifuge (4°C)

Water bath

Parafilm

Tubes 15 X 100 mm (four times the number of samples to be analyzed)

1st series: acido-solubles
2nd series: RNA
3rd series: DNA
4th series: colorimetric dosage

Tubes 13 X 75 mm (for homogenates 1/50) in ice bath

Conical tubes 15 mL in ice bath

Potter 10 or 15 mL

Becher with ice for potter

Pasteur pipettes sealed at one extremity (to be used as agitators)

Acetic acid (CH_3COOH)

Sulfuric acid (H_2SO_4)

Perchloric acid 60% ($HClO_4$)

Diphenylamine reagent

Potassium hydroxide (KOH)

Liquid Scintillation Counter (LS6000TA, Beckman Instruments Inc.)

Scintillation vials

Ecolite

Glass marble

Microplate reader

Solutions preparation (24-48 hours prior to dosage and store at 4°C):

- $HclO_4$ solutions:

Concentration	Required volume
1.2 M	1.2 mL / sample
1.0 M	2.0 mL / sample
0.5 M	1.0 mL / sample
0.3 M	4.0 mL / sample
0.2 M	12.0 mL / sample

$HclO_4$ solution concentration	Required quantity of $HclO_4$ (for 36 samples)	Final volume to obtain with distilled water
1.2 M	6.5 mL $HclO_4$ 60%	43.5 mL
1.0 M	27.5 mL $HclO_4$ 60%	222.5 mL
0.5 M	20.00 mL $HclO_4$ 1.0 M	20 mL
0.3 M	45.00 mL$HclO_4$ 1.0 M	105 mL
0.2 M	90.00 mL $HclO_4$ 1.0 M	360 mL

-KOH solutions:

Concentration	Required volume
0.3 M	2.0 mL / sample

KOH solution concentration	Required quantity of KOH (for 36 samples)	Final volume to obtain with distilled water
0.3 M	30.00 mL KOH 1.0 M	70 mL

-Diphenylamine reagent:

	Required volume
Diphénylamine reagent	2.0 mL/sample (add 24 mL for the standard curve)

Quantity (mL)	Diphenylamine	CH_3COOH (mL)	H_2SO_4 (mL)	Acetaldehyde (mL)
75	1.125	75	1.125	0.375
100	1.50	100	1.50	0.50
150	2.25	150	2.25	0.75
200	3.00	200	3.00	1.00
250	3.75	250	3.75	1.25
300	4.50	300	4.50	1.50

Homogenate preparation:

-Weight about 0.08 g of cortex and put in 2 mL of cold distilled water.

-Homogenize with a small potter in ice.

-Put in tubes identified "DNA homogenates".

-Add DNA dilution 1/50 solution in each tubes.

DNA extraction:

1) 2 mL cortex homogenate 1/50 + 4 ml $HclO_4$ 0.3 M for 10 minutes at 0°C. Mix well with

a sealed Pasteur pipette.

2) Centrifuge 10 minutes (2500 RPM; 0°C). This yield the acido-soluble supernatant fraction.

3) Wash with 3 mL of $HclO_4$ 0.2 M at 0°C. Centrifuge 10 minutes (2500 RPM; 0°C). Take the supernatant fraction and put in tubes identified acido-soluble.

4) Repeat operation 3) one more time.

5) Add 2 mL of KOH 0.3 M, mix well and put in a 37°C water bath for 1h (stir often).

6) Cool in ice.

7) Add 1.2 mL of $HclO_4$ 1.2 M at 0°C and let stand for 10 minutes at 0°C. Centrifuge 10 minutes (2500 RPM; 0°C). Take the supernatant and put in tubes identified RNA.

8) Wash with 3 mL of $HclO_4$ 0.2 M at 0°C. Centrifuge 10 minutes (2500 RPM; 0°C). Take the supernatant fraction and put in tubes identified RNA.

9) Repeat operation 8) one more time.

10) Drain the tubes.

11) Add 2 mL of $HclO_4$ 1.0 M, mix well and put in a water bath at 65°C for 15 minutes (stir three times during this step).

12) Add 2 mL of distilled water. Centrifuge 10 minutes (2500 RPM; 0°C). Take the supernatant fraction and put in tubes identified DNA.

13) Take 0.7 mL of DNA supernatant and put in scintillation vials.

14) Add 10 mL of Ecolite.

15) Proceeds to dosages.

*** Make sure you keep the acido-soluble and the RNA supernatants in the eventuality of problems with dosages.

DNA dosage:

1) Prepare the 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 of solution

2) Prepare the diphenylamine reactant (under the fume hood):

add 700 µl DNA supernatant + 300 µl $HclO_4$ 0.5 M (prepare two tubes of each)

add 2 mL diphenylamine reagent and shake well

cover the tubes with glass marble

incubate (16-20 hours; 30°C)

read at 600 nm with a microplate reader (200 µl of the final product / hole)

DNA standard:

1) Take 5 mL of salmon sperm DNA (Type III) and add 5 mL $HclO_4$ 1.0 M.

2) Under the fume hood, add 2 mL diphenylamine reagent.

3) Shake well.

4) Put in a water bath (15 minutes; 70°C).

5) Prepare two tubes for each DNA standard:

	Standard DNA (µl)	$HclO_4$ 0.5 M (µl)
Blank	-	1000
10	100	900
20	200	800
30	300	700
40	400	600
50	500	500

Calculations:

Cellular regeneration: $\frac{\text{DPM (radioactivity)}}{\text{[DNA]}}$

[DNA]: $\frac{\text{weight DNA*}}{\text{Sample volume (700 µl)}}$

* On the spectrophometer, the concentration read is different from the real concentration. (As instance: The concentration obtained during the dosage is 236 but corresponds to 23.6 µg).

So: [DNA] = $\frac{23.6\ \mu g}{700\ \mu l} = 0.0337$ mg/mL

4.0 Urinary proteins

Material:

BCA Protein Assay Reagent Kit #23225 (Pierce, Rockford, IL, USA)

Borosilicate tubes 13 X 75 mm (one per urine sample)

Pipetman P-200, P-1000, P-10

Plate with 96 holes

Sterile tube 15 mL

Standard capsule

Spectrophotometer

Methods:

1) Dilute urines (1/15) with milli-Q water in 5 ml borosilicate tubes, (i.e: 70µl urine + 980 µl water).

2) Prepare BCA reactant in 15 mL tubes. Put one part of reactant B for 50 parts of reactant A. We need 200 µl of reactant per sample and 2400 µl for the standard curve, (i.e.: for 24 samples we need 8 ml: 160 µl of reactant B + 8 ml reactant A).

3) Thaw samples for the standard curve (200, 400, 600, 800, and 1000).

4) Put 10 µl of each samples in their respective hole on the plate of 96 holes.

A	200	200	5
B	400	400	6
C	600	600	7
D	800	800	8
E	1000	1000	9
F	water	water	10
G	1	3	11
H	2	4	12

5) Put 200 µl of BCA reactant in each hole.

6) Incubate for 30 minutes at 37° in a water bath.

7) After 30 minutes, remove the plate of the water bath and verify the coloration of the standard curve.

8) Read the plate with a spectrophotometer:

9) Verify correlation with the standard curve (>0.99).

Standard curve:

Standard volume	Water volume	Final concentration
500 µl (stock)	500 µl	1000µg/mL
400 µl (stock)	600 µl	800 µg/mL
300 µl (1000)	200 µl	600 µg/mL
200 µl (800)	200 µl	400 µg/mL
200 µl (400)	200 µl	200 µg/mL

Calculations:

- Concentration obtained * 10 (instrument factor) * (dilution factor) = µl/mL

- µl/mL * diuresis = µg/24-h / 1000 = mg/24-h

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