AD-A182 033 THE EFFECTS OF CORTISOL CORTICOSTERONE INSULIN AND GLUCOSE PRE- AND POST-..(U) TUSKEGEE UNIV AL CARVER RESEARCH FOUNDATION M E TOLBERT ET AL. 27 DEC 86 1/2

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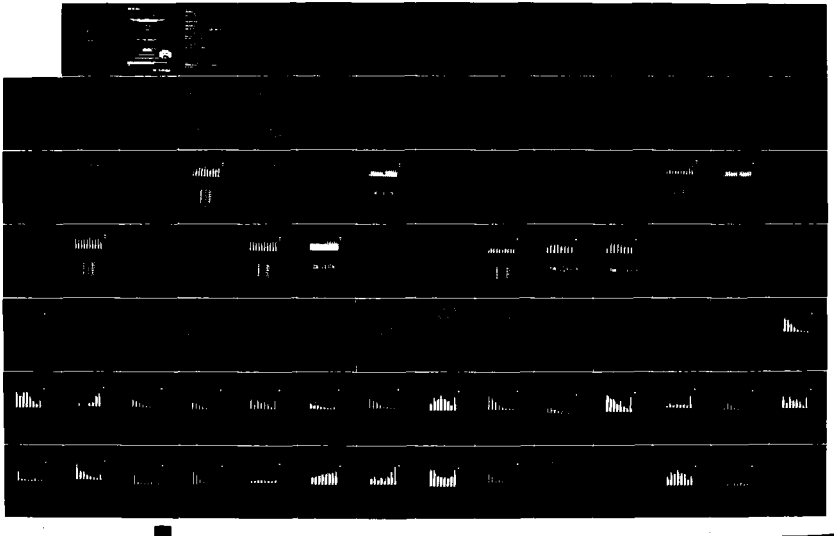

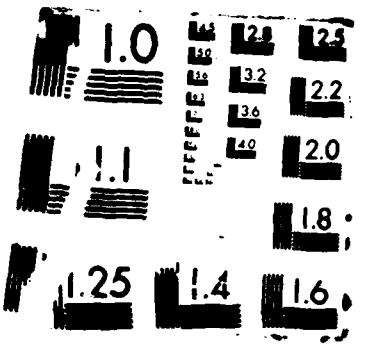
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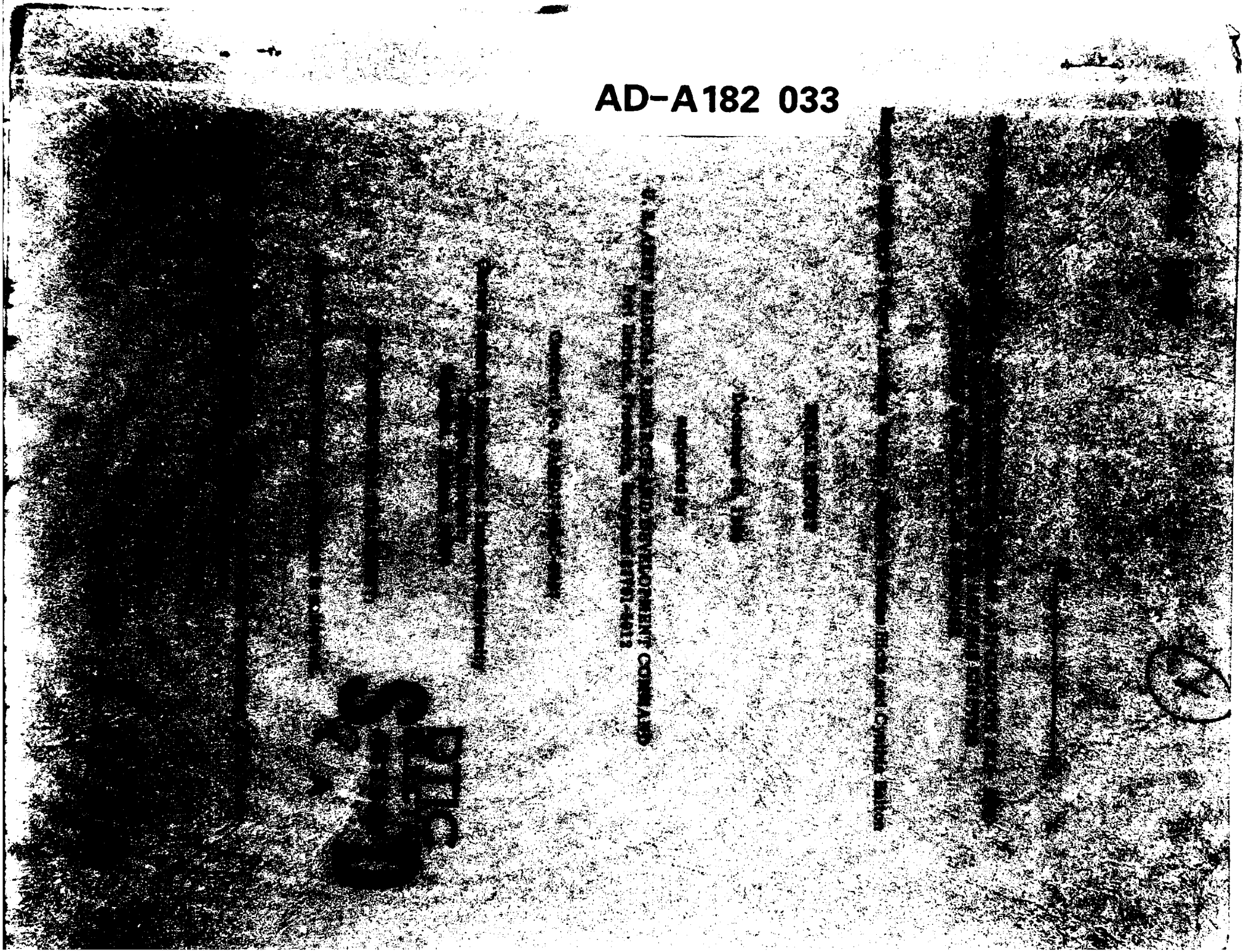

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12. PERSONAL AUTHOR(S): Tolbert, Margaret Ellen Mayo; Weaver, Abraham; Hicks, Vernetta Echoles; Belton, Cynthia

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In studies of heat stress, a series of in vivo and in vitro experiments were designed to determine if mixtures of either cortisol or corticosterone plus insulin and glucose have beneficial effects on hyperthermic rats. Hyperthermia was induced in male rats (450 to 550 grams) by exposure to severe heat loads (42°C to 42.6°C). The rats were then allowed to cool to or below 40.4°C. Blood samples were taken at this point or after 24 hours of survival depending upon the experimental protocol. Endogenous corticosterone, LDH, SGOT and potassium levels increased immediately. In the 24-hour viability studies, rats were injected after heat stress with post-treatment regimens of corticosterone, insulin and glucose in solution. Our results show that the number of control rats (15%) which died after heat exposure was three times greater than the number which had been post-treated (5%) after heat exposure. Studies using isolated hepatocytes showed that a mixture of corticosterone, insulin and glucose caused minor reductions in the SGOT and LDH levels due to hyperthermia.

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Further investigations were conducted using I-125 labeled corticosterone to study the biodistribution patterns of the radiolabeled compound in the heat-stressed and unheated control animal model. There was a time course body distribution pattern of the i.v. administered radiolabeled compound noted in which the heat-stressed animals demonstrated a faster uptake and longer retention time of I-125 labeled corticosterone than did the unheated control animals.

The rat livers were further analyzed for I-125 labeled corticosterone content in order to identify liver subcellular localization patterns. Rapid uptake and longer retention times were characteristics of the radiolabeled whole liver homogenates of the heat-stressed rats. Nuclear and soluble cytosol fractions of these homogenates also demonstrated a more rapid uptake and longer retention time of the radiolabeled compound as compared to the unheated control rats.

Our results ~~seem to~~ show that i.v. post-treatment of the rats with the indicated drug mixture is valuable in treating heatstroke.

SUMMARY

Hyperthermia was induced in male rats (450 to 550 grams) by exposure to severe heat loads (42.2°C to 42.6°C). The rats were then allowed to cool passively to or below 40.4°C. Blood samples were taken at this point or after the rats had survived 24 hours, depending upon the experimental protocol. Endogenous corticosterone, LDH, SGOT and potassium levels increased immediately after the animals were exposed to heat stress. Hyperthermic rats distributed intravenously administered I-125-corticosterone somewhat differently than did unheated control rats. Organ and urine samples from hyperthermic rats retained more of the radiolabeled compound after 30 to 60 minutes then did the control rats. In the 24-hour viability studies, rats were injected with post-treatment regimens of corticosterone, insulin and glucose in solution. It appears that post-treatment with this mixture is a valuable tool in treating heatstroke. In efforts to delineate the functions of a corticosterone, insulin and glucose mixture on liver, the isolated hepatocyte system was used in a series of studies. The mixture caused minor reductions in the SGOT and LDH levels of hyperthermic hepatocytes.

Further investigations were done to evaluate the effects of heat stress on glucose production from isolated hepatocytes. There was a significant increase in glucose production when the cells were incubated at 37°C or 42°C in 10 mM lactate in which a corticosterone-insulin or cortisol-insulin drug regimen had been added as compared to the incubation in 5 mM glucose or without glucose at 30 minutes. However, at the 60 or 120 minute incubation periods there were no significant differences measured for the drug treatments in the various incubation media.

Further investigations were conducted using I-125 labeled corticosterone to study the biodistribution patterns of the radiolabeled compound in the heat-stressed and unheated control animal model. There was a time course body distribution pattern of the i.v. administered radiolabeled compound noted in which the heat-stressed animals demonstrated a faster uptake and longer retention time of I-125 labeled corticosterone than did the unheated control rats.

The livers of the I-125 corticosterone injected rats (either heat-stressed or controls) were processed for subcellular liver fraction studies. There was a rapid uptake and longer retention time of the radiolabeled compound in the total liver homogenates and soluble fractions in the heat-stressed animals as compared to the unheated controls. Therefore, the results in this report demonstrate that heat stress will affect various metabolic and enzymatic processes throughout the body and that the previously indicated drug treatment design might be a useful tool in treating heat stress, but the actual mechanisms involved are unclear.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

This report is submitted in reference to contract DAMD 17-86-C-6087 and in accordance with the August 7, 1986 letter (Ref.: SGRD-RMS) from the Scientific and Technical Information Office of The Department of The Army, Fort Detrick, Maryland.

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PROJECT STAFF

Miss C. E. Belton, Chemist, 100% Time for 6 months (Part of her salary for this period was paid by the Carver Research Foundation of Tuskegee University).

Dr. U. D. George, Biometricist, 20% Time ended September 30, 1986

Mrs. V. E. Hicks, Chemist, 100% Time

Ms. A. Mauldin, Biologist, (Not paid with funds from this project.)

Dr. S. Sodeke, Medical Technologist, (Not paid with funds from this project.)

Dr. M. E. M. Tolbert, Biochemist, 10% Time

Dr. A. Weaver, Radionuclear Pharmacist, 100% Time

COMMENTS ON ADMINISTRATIVE AND LOGISTICAL MATTERS

During the pre-contract period (August 8, 1985), Dr. Lawrence E. Armstrong of USARIEM and Ms. Lisa K. Griffin of the U. S. Department of the Army visited the Carver Research Foundation and discussed details pertinent to the proposal application. Notification of approval was received in October 1985. After assignment of an account number by the Tuskegee University Office of Grants Management, project staff positions were advertised and one full-time Research Assistant and a part-time Research Associate were hired. Requisitions were prepared for the purchase of equipment, supplies and renovation services. The required approvals for the above were received from the contracting agency (Ref.: Letters dated November 25, 1985 and February 5, 1986, from Ms. Griffin and Ms. Shinbur, respectively).

By the time that the first project report was submitted, the animal room, located in the basement of the Carver Research Foundation Building, had been renovated. The research laboratory located in Room 10 of the same building had been equipped with laboratory furniture and utilities. The Tuskegee University Animal Care Committee had approved of the facilities for handling and housing the experimental animals to be used (Appendix 1). The Laboratory Animal Services Unit of the School of Veterinary Medicine, Tuskegee University, had approved of the experimental procedures to be used in the project (Appendix 2). Copies of the approved documents (Appendices 1 and 2) were sent to Dr. Lawrence E. Armstrong, the COTR at USARIEM; Dr. George Silver chairman of USARIEM Animal Use Committee; and Dr. Wilbert Bowers project collaborator at USARIEM.

In conducting the research described in the first semi-annual report, the investigators followed guidelines which appear in "Guide for Laboratory Animal Facilities and Care" (as promulgated by the Committee of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council).

In order to conduct the research, which involved the heating of rats, Dr. Tolbert and Mrs. Hicks spent 16 days (February 22 to March 9, 1986) and 9 days (February 26 to March 6, 1986), respectively, conducting research, completing related tasks at USARIEM in Natick, Massachusetts, and traveling to and from USARIEM.

In addition to daily conferences with Dr. W. Bowers of USARIEM (project collaborator and the person in whose laboratory Dr. Tolbert and Mrs. Hicks conducted research), the following persons were among those with whom Dr. Tolbert held discussions pertinent to the project while at USARIEM: Col. B. Joyce, Dr. L. E. Armstrong (COTR), Dr. M. Durkot, Cpt. Bruttig, Dr. G. Silver (Director of Animal Facility), Mr. T. Martin (Radiation Safety Officer) and Ms. C. B. Matthew.

Samples of serum and tissue collected during the completion of the project research conducted at USARIEM were frozen and transported to the Carver Research Foundation of Tuskegee University for analyses.

During the second six-month period (April 28, 1986 to October 28, 1986) of the contract, the project director kept in constant contact with Dr. Lawrence E. Armstrong (COTR) and Dr. Wilbert Bowers (collaborator) at USARIEM. Of primary concern was the receipt of authorization for the use of radioisotopes and equipment which produces ionizing radiation and the approval of the protocol by the USARIEM Laboratory Animal Care and Use Committee (LACUC). The project protocol had already been approved by the Tuskegee University Radiation Safety Committee which provided authorization for that facet of the research which involved the utilization of various radioactive isotopes. Prior to this period, the project director had not been apprised of the need for a review by USARIEM LACUC; however, she considered it of great value since some aspects of the research were to take place at USARIEM.

The USARIEM LACUC reviewed the research protocol in May 1986 at USARIEM, and in June it recommended several technical revisions. In July, the project director responded as shown in Appendix 3. In reference to this response (Appendix 3), a notice of approval was received in August (Appendix 4).

A document which contains details on the preparation and processing of medical research and development reports was received in August 1986. This document was provided by the Scientific and Technical Information Office of the Department of the Army at Fort Detrick.

On September 26, 1986, the notice (Modication No. P60002) of additional funds in the amount of $19,954 for supplies, services and travel was received. These funds were intended to cover the budget increase due to revisions recommended by USARIEM LACUC. The latter part of September and early December were spent in preparation for the second trip to USARIEM. Supplies were ordered, permission was obtained from USARIEM for the visit and the use of the heating facility and selected items of equipment.

On October 15, 1986, Major Theodore Allen from the Medical Research and Development Committee (Department of the Army, Fort Detrick) conducted a site visit at the Carver Research Foundation of Tuskegee University. He was informed of the on-going research and the plans for our December 1986 visit to USARIEM. He also presented a very informative seminar on the various research opportunities and sources of funding from the Department of the Army.

In order to complete the remaining research, a budgetary shift was requested by Dr. Tolbert. The request was based on the need for more supplies. No additional funds were requested. Funds needed were available in the travel category. In the first week of December, Dr. Tolbert, Dr. Weaver, Mrs. Hicks and Miss Belton went to USARIEM to complete those experiments which required the heating of whole animals. A malfunctioning computer system caused the loss of several rats; however, the team was able to complete the experiments by December 19, 1986. Some liver samples were taken back to Tuskegee University for analysis of radiolabeled contents.

While at USARIEM, Dr. Tolbert met with Col. Schnakenberg (Commander of USARIEM) to discuss the project. He advised her of his well wishes in having this joint research venture and offered his continued support. Among the other persons with whom the project was discussed were Dr. Armstrong, Dr. Bowers, Dr. Francisconi, Dr. Hubbard and a host of other scientists at USARIEM.

SUMMARY OF APPROXIMATE PRINCIPAL INVESTIGATOR EXPENDITURES OR COMMITMENTS ON THE CONTRACT

Expenditures and Commitments

Personal Salaries + Fringe Benefits	$ 51,676.00
Equipment	$ 25,970.00
Supplies	$ 30,383.00
Travel	$ 7,134.00
Renovations	$ 10,500.00
Overhead	$ 26,758.00
Total Amount	$152,421.00

BRIEF STATEMENT OF PLANS FOR THE FUTURE

It is anticipated that a continuation contract will be awarded and that the research will be continued.

Scientific Progress

October 1985 — December 1986

THE EFFECTS OF CORTISOL, CORTICOSTERONE, INSULIN AND GLUCOSE PRE- AND POST-TREATMENT ON HEATSTROKE IN RATS AND THE KINETICS OF UPTAKE AND CELLULAR RESPONSE

STATEMENT OF THE PROBLEM

Methods to prevent or reduce the number of heatstroke fatalities are being sought by various sectors of society. Heatstroke is of concern to the military which often has to rapidly mobilize troops for exercises in tropical, sub-tropical and desert areas. The problem is further amplified by the potential requirement for use of vehicles with no cooling capacity and the use of certain drugs. United States service personnel could operate more effectively in hot weather if they had the information, training and equipment required to adjust work-rest cycles and water intake to prevailing environmental conditions. In cases where exposure to excessive heat load may be unavoidable and heat illness imminent, an effective post-treatment regimen may have beneficial effects, subsequent to heat exposure, and could serve a valuable function since the mortality rate from heatstroke could be as high as 80%.

In efforts to find solutions to the problems of heatstroke, a number of studies have been designed and implemented using the rat as the experimental animal model. A large number of these studies have been conducted or are in progress at the United States Army Research Institute of Environmental Medicine (USARIEM). Studies on the use of a pre-treatment regimen (cortisol, insulin and glucose given intraperitoneally) for the prevention of heatstroke in rats were conducted by Dr. W. D. Bowers and co-workers at USARIEM in 1984. A member of the present research team, Mrs. V. E. Hicks, participated in that research. Results from those efforts suggest that pre-treatment does not increase 24-hour viability in rats which have been heated to heatstroke temperatures.

In our present work, we used a different approach in which we administered various drugs (intravenously) as a post-treatment drug regimen after heat stress in an attempt to preserve the cellular integrity of the heat-stressed rat model. Considerable evidence supports the validity of this new approach. The capacity to reduce ischemic damage with glucocorticoids has been well documented, and glucose, insulin and potassium (GIK) are used extensively in the treatment of myocardial infarctions. GIK increases arachidonic acid formation, influences phospholipid membrane stabilization and increases prostaglandin formation. All of these effects are beneficial in reducing necrosis. GIK also increases glycolysis, decreases free fatty acids (FFA) and changes FFA composition. These changes improve ATP production. According to some investigators, the normal heart derives a substantial fraction of its energy from fatty acids and a negligible fraction from anaerobic glycolysis. However, the latter fraction increases progressively as the heart is rendered anoxic, as the perfusion medium is enriched with glucose and as insulin is added. The membrane barrier to glucose entry into the cell is overcome by high extra-cellular glucose and insulin. GIK also restores intracellular potassium and insulin stimulates glycogen synthetase.

Injury due to myocardial ischemia and that due to heatstroke share several common sequelae which provide further evidence tending to validate the present approach. The earliest change in either case is believed to be loss of membrane integrity. Cardiovascular failure may determine lethality in either case. Reversal of K+ leakage also improves tissue integrity. With these facts in mind and in a natural progression of previous research, we post-treated rats with insulin, glucose and corticosterone or cortisol after exposing them to a severe heat load to determine whether or not this post-treatment improves 24-hour viability. We also studied the responses of isolated hepatocytes to heat, with or without hormone treatment.

HYPOTHESIS

Insulin, glucose and cortisol or corticosterone given together as a post-treatment regimen may reduce heat injury to cells and thus improve the survival of rats after a heat exposure which would ordinarily produce a high rate of fatalities in 24 hours. This hypothesis is based on the beneficial use of insulin and cortisol in heat injury to the isolated perfused liver, the successful use of glucose, insulin and K+ in treating ischemic injury to the heart after myocardial infarction, current theories on a stepwise progression leading to irreversible cell injury and the use of the rat as an experimental model for studies on heatstroke mortality (18). Benefits attributed to stabilizing membranes are applicable in each case.

STATEMENT OF MILITARY RELEVANCE

According to Hubbard et al. (16), heat casualty rates remain high among military units participating in combat exercises in tropical and subtropical areas and in the Mojave Desert. Their analysis suggested that U. S. Armed Forces could operate effectively in hot weather if commanders had the information, training and equipment required to adjust work-rest cycles and water intake to prevailing environmental conditions. In actual combat situations, the obduracy of the enemy could be the overriding factor determining both the work-rest cycles and the availability of water. In such cases, where exposure to excessive heat load may be unavoidable and heat illness imminent, an effective post-treatment may bring about beneficial effects subsequent to heat exposure and could serve a valuable function since the mortality rate for heatstroke could be as high as 80% (23).

STATISTICAL ANALYSIS OF EXPERIMENTAL DATA

The data in this report was analyzed by Two-Way Analysis of Variance (ANOVA) using the Statistical Analysis System (SAS) with differences being regarded as significant when $p < 0.05$. Further statistical analyses were performed using the t-test and Least Significant Difference (L.S.D.) comparison test between each treatment group with differences being regarded as significant when $p < 0.05$. SAS programs were also implemented to obtain the means and standard error of the means of each treatment group. Graphic comparisons of each treatment group were obtained using the Graphwriter computer package.

A. RESPONSE OF ISOLATED HEPATOCYTES TO HEAT AND HORMONES

THE SETTING

Experiments designed to address the characterization of isolated rat hepatocyte responses to heat and hormones were conducted at the Carver Research Foundation of the Tuskegee University.

EXPERIMENTAL METHODS

Hepatocytes were isolated from male Sprague Dawley rats (450 to 550 grams) as outlined in Appendix 3 and diluted to 50 mg/ml. The isolation procedure used was a modification of those by Berry and Friend (2) and Johnson et al. (20). Aliquots of the hepatocyte suspensions were assayed for protein content (Appendix 3, Attachment 9). Hepatocytes (with or without a glucocorticoid, insulin or insulin/glucocorticoid mixtures; glucocorticoid=cortisol or corticosterone) were incubated with shaking at either 37°C or 42°C for the desired periods in plastic test tubes. At the end of the incubation periods, samples were chilled in an ice/water bath and centrifuged. Supernatants were separated from the cell pellets. Supernatants were analyzed for glucose, SGOT, K+, Na+ and LDH levels with the aid of an autoanalyzer (Technicon SMA II or the Flexigem). In cases when only glucose measurements were needed, the glucose oxidase method was used (Appendix 3, Attachment 8). Glycogen determinations were made by the method outlined in Appendix 3, Attachment 10.

Corticosterone hemisuccinate BSA was purchased from Sigma. Cortisol was purchased from the Upjohn Company and porcine insulin was a gift from Eli Lilly Company.

RESULTS

Isolated hepatocytes that had been exposed to various drug treatments (corticosterone, cortisol, insulin or glucose) at either 37°C or 42°C incubation temperatures for either zero or 30 minutes demonstrated varied responses. These studies were conducted to determine if a mixture of glucose, corticosterone and insulin, as well as a mixture of glucose, cortisol and insulin, would affect either the enzymatic levels of lactate dehydrogenase (LDH) and supernatant glutamic oxaloacetate transaminase (SGOT) or the levels of sodium and potassium ions in the supernatants of the isolated hepatic parenchymal cell system used in our laboratory. LDH levels increased significantly ($p < 0.04 - p < 0.004$) in the supernatants of the isolated hepatocytes (Table 1, 3, Figure 1) with increases in the incubation time and temperature (0 or 30 minutes and 37°C or 42°C, respectively). There was a significantly ($p < 0.04 - p < 0.0001$) greater LDH release measured when the cells were incubated at either 37°C or 42°C in

5 mM glucose as compared to the cells incubated without glucose (Tables 1 and 3, Figure 1). The cells incubated at 42°C for 30 minutes demonstrated significantly ($p < 0.05$) higher LDH levels when compared to the cells incubated at 37°C in the same drug mixtures. However, the LDH levels did not exceed 1350 ± 99 U/L at 30 minutes (Tables 1, 3, Figure 1). The hepatocytes incubated at 42°C appeared to be more sensitive to the presence of the corticosterone, insulin and glucose mixture than to the cortisol and insulin mixture (Tables 1 - 3, Figures 1 - 2). Due to the greater sensitivity of the rat hepatocytes to corticosterone than to cortisol and since rats have several fold more endogenous corticosterone than cortisol, only the effects of corticosterone were measured at longer incubation time periods (Tables 2, 4, 6, 8). There was a somewhat greater leakage of LDH from the cells when 5 mM glucose was added to the cell suspensions than in the absence of glucose; however, the drug mixture appeared to work better in the presence of glucose and might indicate that corticosterone, at 33 - 49.5 mg/ml, activates various cellular protective mechanisms against either drug toxicity or heat stress. When the cells were incubated at 42°C for either 60 or 120 minutes (Tables 2, 4, Figure 2), the release of LDH was reduced approximately 5% and 4%, respectively, in the presence of a mixture of corticosterone (3.3 - 33 ug/ml) plus insulin and 5 mM glucose. In the presence of 10 mM lactate (Tables 2, 4, Figure 2), the LDH released by the control hepatocyte suspensions was not significantly greater than the LDH released in the presence of 5 mM glucose (Table 4, Figure 2). The reduction in LDH released from the liver cell suspensions incubated in a mixture of corticosterone, insulin and 10 mM lactate was greater than that measured in the presence of 5 mM glucose (Table 4, Figure 2) when the cells were incubated for 60 or 120 minutes.

When the hepatocytes were incubated in the absence of any added drug, there was a significantly ($p < 0.05$) greater release of SGOT from the cells into the incubation medium at 42°C than at the 37°C incubation temperature (Tables 5 - 8, Figures 3 - 4). In the presence of 5 mM glucose, the levels of SGOT release are comparable for the corticosterone and cortisol treated samples. However, in the absence of glucose, the levels of SGOT released were higher at 42°C than at 37°C in a mixture of corticosterone and insulin as compared to the cortisol and insulin mixture (Table 7, Figure 3). There was a sustained elevation of the SGOT levels at 42°C even when the cells were incubated in a mixture of glucose, cortisol and insulin (Table 7, Figure 3). However, mixtures of corticosterone, insulin and glucose reduced the amount of SGOT released into the media after 30 minutes at 42°C (Figure 3). This reduction was also produced when corticosterone was replaced with cortisol in the mixture (Figure 3).

There was a small reduction from basal levels in the SGOT released when the cells were incubated in the presence of a mixture of corticosterone, 5 mM glucose and insulin (1.0 uU/ml) at either 60 or 120 minutes (Tables 6, 8, Figure 4). However, none of the reductions were greater than 15% and were not significantly different (Tables 6, 8, Figure 4).

The effects of glucose, insulin, corticosterone and cortisol mixtures on the Na+ levels in the hepatocyte suspensions are shown in Tables 9 - 10, Figure 5. There was a significant ($p < 0.02 - p < 0.06$) decrease in the sodium (Na+) levels in the liver cell supernatants when the cells were incubated in the presence of 5 mM glucose (with or without the drug mixture) at either 37°C or 42°C in most of these investigations.

In the absence of any exogenously added glucose in the incubation medium, the potassium (K+) levels (Tables 11, 13, Figure 6), like Na+ (Tables 9 - 10, Figure 5), were significantly ($p < 0.05$) higher than those noted for the K+ levels measured when 5 mM glucose was added to the incubation medium and the cells incubated at 37°C or 42°C for 30 minutes. However, the presence or absence of cortisol or corticosterone had no effect when compared to the controls in the presence or absence of glucose.

It seems that the presence of added K+ in the incubation medium may have prevented any major cellular fluxes of the K+ levels. At the 37°C or 42°C incubation temperatures, in which the cells were incubated either 60 or 120 minutes, there were no significant differences noted in the K+ levels in the cells incubated in the various drug mixtures (Tables 12, 14, Figure 7). Further experiments are planned to address a protocol in which K+ is deleted from the incubation buffer. Perhaps, this deletion, will cause greater K+ fluxes which can be detected under our experimental conditions.

When the isolated hepatocytes were incubated for 30 minutes in the presence or absence of insulin (1.0 uU/ml) plus increasing concentrations of corticosterone or cortisol (33.3 - 49.5 ug/ml) and 10 mM lactate, there was a significant $p < 0.05$ increase in the amount of glucose produced as compared to either the 5 mM glucose addition or no glucose at 37°C and 42°C (Figure 8). There was a significant ($p < 0.05$) decrease in glucose production when the cells were treated with insulin (1.0 uU/ml) plus cortisol or corticosterone (33 - 49.5 ug/ml); however, the presence of corticosterone in the medium caused the greatest decreases in glucose production compared to the presence of cortisol (Figure 8). Therefore, these results seem to demonstrate that the isolated liver cell system is more sensitive to corticosterone than to cortisol when compared under the same conditions. Also, the results show that cells can produce glucose from lactate, an indication of viability.

In the presence of exogenous glucose (5 mM) or lactate (10 mM), the over-all glucose production responses of the isolated cells to these agents were similar (Figures 9 - 10) at either 37°C or 42°C. When the cells were incubated in the presence of high levels of corticosterone and corticosterone plus insulin, less glucose was produced at 60 and 120 minutes when compared to the controls (Figures 9 - 10).

In a separate series of experiments, cellular responses of the isolated liver cells were tested in the absence of insulin and corticosterone (Figure 11). In an overall comparison of these results it was demonstrated that at 37°C or 42°C the presence of 10 mM lactate in the incubation medium would cause the cells to increase glucose production with a corresponding decrease in glycogenolysis (Figures 8 - 11). These findings also demonstrate the suitability of our test models since the cells seem to be still viable and to be undergoing all of their metabolic processes at either a reduced or accelerated rate.

Table 1. Effects of Insulin, Glucose, Corticosterone and Cortisol on Lactate Dehydrogenase Levels in Isolated Hepatocytes Incubated at 37°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		37°C	37°C	Difference
Control	0	528± 71*[c]	837± 43*[c]	P < 0.004
Control	30	802± 63[b]	1075± 67[ab]	P < 0.02
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	801±117[b]	1133± 72[ab]	P < 0.04
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	1007± 91[ab]	1106± 81[ab]	N. S.
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	973± 72[ab]	1257± 92[a]	P < 0.04
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	943± 70[ab]	1256±113[a]	P < 0.04
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	910±115[ab]	980±101[bc]	N. S.
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	1028±127[ab]	1094± 58[ab]	N. S.
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	967± 68[ab]	1124± 33[ab]	P < 0.06
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	1078± 92[a]	1163± 73[ab]	N. S.

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in units per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

(a, b, c) Any two means within a column with the same superscript are not significant at the five percent level of probability using the LSD test. LSD at 0.05 = 261.18 or 220.09 when the cells were incubated without glucose or in 5mM glucose, respectively.

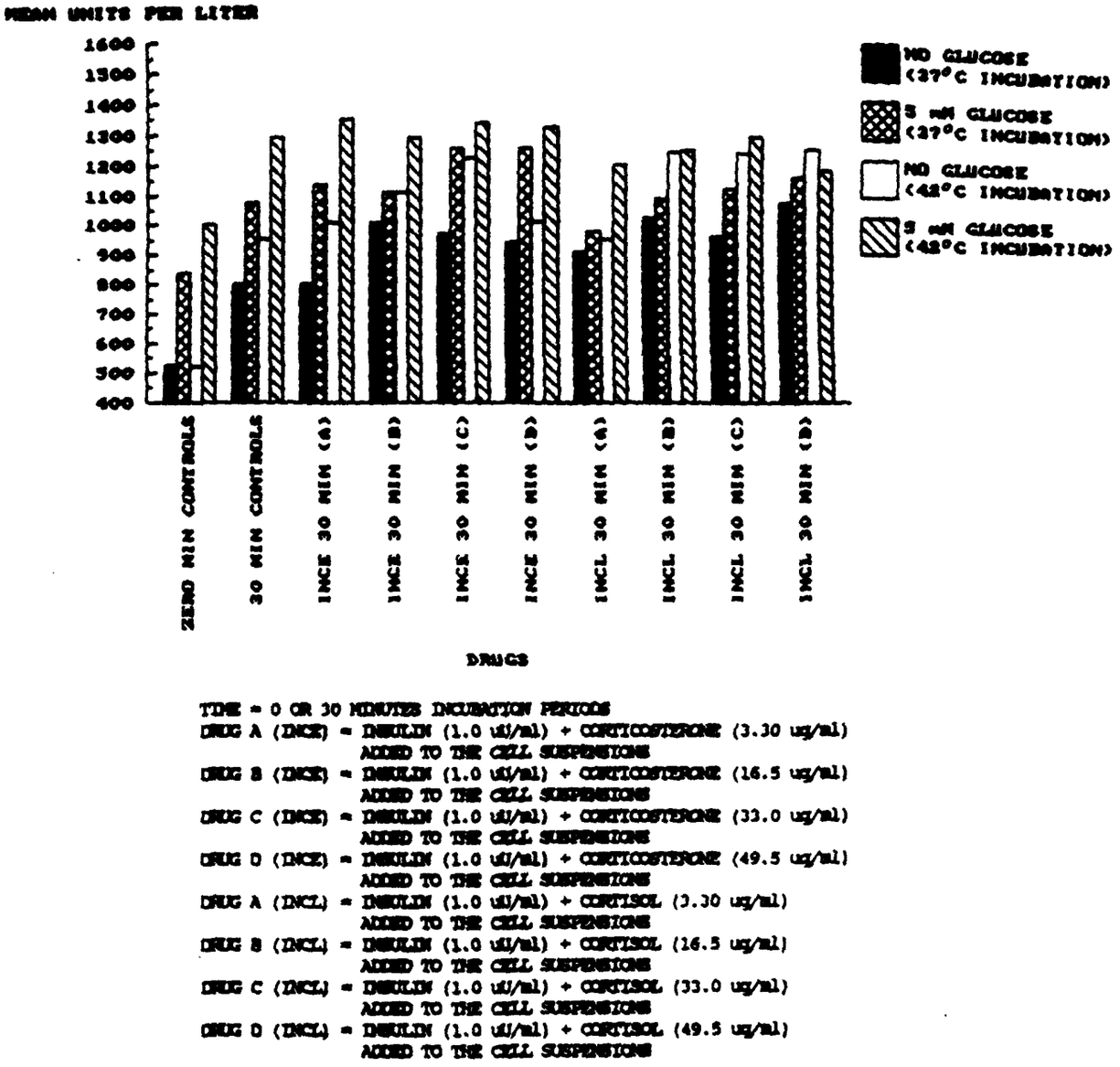
(1) Probability of difference based on t test. N. S., not significant at five percent.

Table 2. Effects of Insulin, Glucose, Corticosterone and Lactate on Lactate Dehydrogenase Levels in Isolated Hepatocytes Incubated at 37°C.

Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	2052 ± 179	1955 ± 160	1867 ± 129
0.0	60	2805 ± 406	2855 ± 333	2386 ± 255
IN	60	2915 ± 420	2795 ± 249	2333 ± 255
3.3	60	3002 ± 325	2808 ± 278	2318 ± 260
16.5	60	2810 ± 368	2888 ± 317	2296 ± 247
33.0	60	2718 ± 357	2665 ± 306	2279 ± 261
49.5	60	2732 ± 401	2768 ± 318	2398 ± 341
49.5 + IN	60	2845 ± 453	2680 ± 279	2456 ± 293
0.0	0	2052 ± 179	1955 ± 160	1867 ± 129
0.0	120	2988 ± 340	3078 ± 284	2686 ± 214
IN	120	2896 ± 305	3068 ± 347	2633 ± 209
3.3	120	3063 ± 329	3019 ± 460	2670 ± 247
16.5	120	2846 ± 394	3141 ± 316	2460 ± 92
33.0	120	2851 ± 394	3139 ± 213	2705 ± 258
49.5	120	2896 ± 405	2939 ± 279	2936 ± 408
49.5 + IN	120	2725 ± 370	2806 ± 327	2773 ± 304

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN) or corticosterone. The values shown are expressed in units per liter ± the standard error of the means of three experiments in duplicate. The exogenous glucose and lactate concentrations were 5 mM and 10 mM, respectively. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.

(a, b, c) Any two means within a column with the same superscript are not significant at the five percent level of probabilty using the LSD test. LSD at 0.05 = 544.54.



TIME = 0 OR 30 MINUTES INCUBATION PERIODS
DRUG A (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG A (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 1. Effects of Various Insulin, Corticosterone and Cortisol Concentrations on Lactate Dehydrogenase Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 30 Minutes with 5 mM Glucose or without Glucose.

Table 3. Effects of Insulin, Glucose, Corticosterone and Cortisol on Lactate Dehydrogenase Levels in Isolated Hepatocytes Incubated at 42°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		42°C	42°C	Difference
Control	0	520± 26*[b]	1003± 52*[a]	P < 0.0001
Control	30	954±109[a]	1296± 94[a]	P < 0.04
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	1006±142[a]	1350± 99[a]	P < 0.08
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	1109±135[a]	1289±156[a]	N. S.
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	1226±146[a]	1342±151[a]	N. S.
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	1014±102[a]	1324±107[a]	P < 0.06
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	953±127[a]	1205±128[a]	N. S.
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	1247±102[a]	1253±115[a]	N. S.
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	1243±126[a]	1293± 58[a]	N. S.
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	1254±195[a]	1184± 53[a]	N. S.

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in units per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

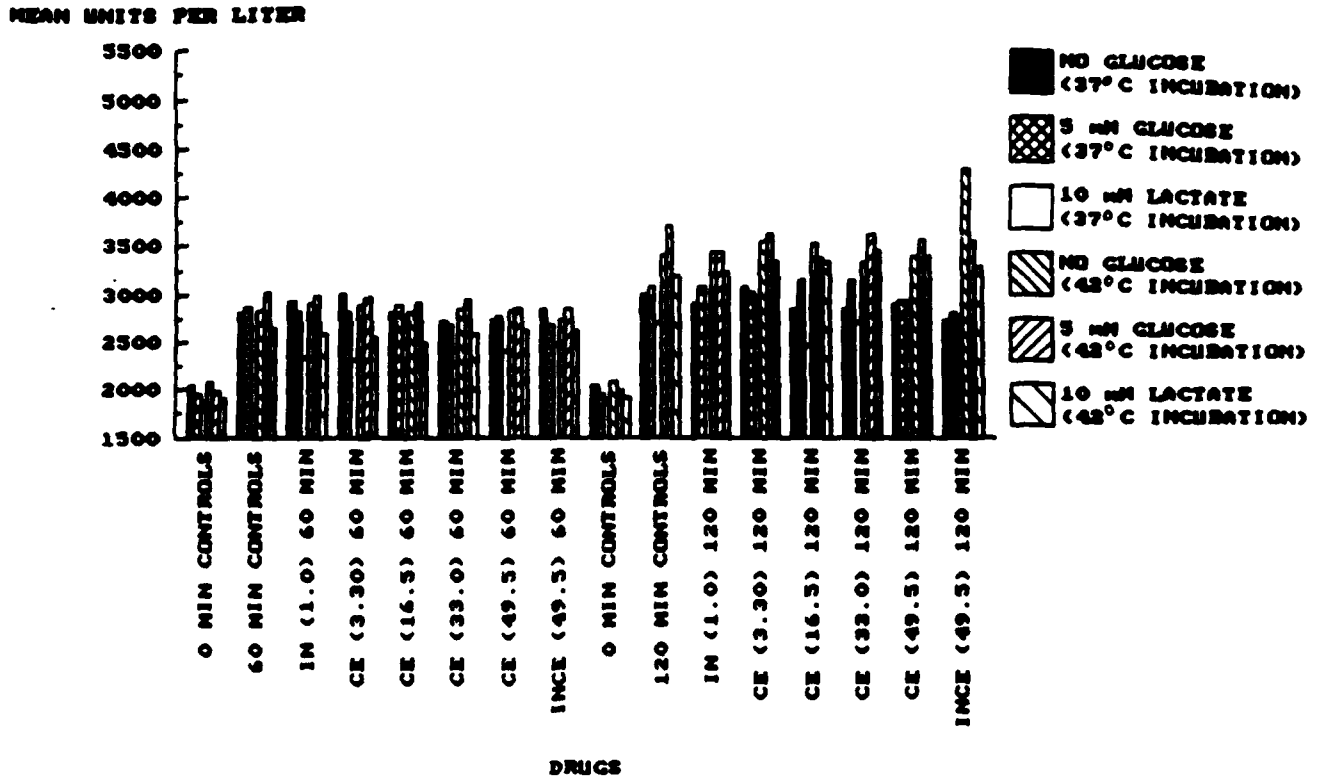
(a, b) Any two means within a column with the same superscript are not significant at the five percent level of probability using the LSD test. LSD at 0.05 = 365.01 when the cells were incubated without glucose.

(1) Probability of difference based on t test. N. S., not significant at five percent.

Table 4. Effects of Insulin, Glucose, Corticosterone and Lactate on Lactate Dehyrogenase Levels in Isolated Hepatocytes Incubated at 42°C.

Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	2080 ± 203	1990 ± 202	1903 ± 166
0.0	60	2826 ± 379	3005 ± 295	2638 ± 303
IN	60	2905 ± 409	2985 ± 286	2579 ± 258
3.3	60	2876 ± 440	2961 ± 254	2544 ± 285
16.5	60	2814 ± 448	2896 ± 278	2479 ± 297
33.0	60	2848 ± 380	2934 ± 300	2569 ± 260
49.5	60	2821 ± 408	2840 ± 236	2611 ± 242
49.5 + IN	60	2724 ± 395	2840 ± 253	2620 ± 226
0.0	0	2080 ± 203	1990 ± 202	1903 ± 166
0.0	120	3388 ± 295	3696 ± 230	3176 ± 296
IN	120	3430 ± 301	3439 ± 244	3216 ± 331
3.3	120	3541 ± 418	3626 ± 384	3335 ± 227
16.5	120	3529 ± 343	3376 ± 277	3340 ± 189
33.0	120	3339 ± 377	3616 ± 410	3445 ± 322
49.5	120	3403 ± 374	3566 ± 507	3385 ± 259
49.5 + IN	120	4285 ± 1029	3544 ± 430	3280 ± 215

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN) or corticosterone. The values shown are expressed in units per liter ± the standard error of the means of three experiments in duplicate. The exogenous glucose and lactate concentrations were 5 mM and 10 mM, respectively. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.



TIME = 0, 60 OR 120 MINUTES INCUBATION PERIODS
IN = INSULIN (1.0 uU/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
INCE = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 2. Effects of Various Insulin and Corticosterone Concentrations on Lactate Dehydrogenase Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0, 60 or 120 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.

Table 5. Effects of Insulin, Glucose, Corticosterone and Cortisol on Supernatant Glutamic Oxaloacetic Transaminase Levels in Isolated Hepatocytes Incubated at 37°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		37°C	37°C	Difference
Control	0	390 ± 56*[c]	378 ± 72*	N. S.
Control	30	509 ± 33[c]	463 ± 80	N. S.
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	453 ± 40[c]	458 ± 83	N. S.
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	472 ± 35[c]	450 ± 85	N. S.
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	468 ± 34[c]	499 ± 102	N. S.
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	478 ± 37[c]	443 ± 86	N. S.
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	492 ± 40[c]	445 ± 80	N. S.
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	527 ± 31[c]	438 ± 80	N. S.
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	698 ± 50[b]	479 ± 94	P < 0.0001
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	987 ± 129[a]	439 ± 50	P < 0.0001

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN), corticosterone (CE) and cortisol (CL), as noted above. The values are the means in units per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

(a, b, c) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

(1) Probabilty of difference based on t test. N. S., not significant at five percent. LSD at 0.05 = 159.84.

Table 6. Effects of Insulin, Glucose, Corticosterone and Lactate on Supernatant Glutamic Oxaloacetic Transaminase Levels in Isolated Hepatocytes Incubated at 37°C.

Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	589 ± 10	478 ± 10	539 ± 11
0.0	60	1087 ± 39	978 ± 85	1098 ± 43
IN	60	1077 ± 31	998 ± 49	1036 ± 22
3.3	60	1215 ± 130	999 ± 18	1016 ± 27
16.5	60	980 ± 10	1025 ± 38	874 ± 112
33.0	60	983 ± 49	983 ± 19	934 ± 36
49.5	60	1012 ± 44	1041 ± 111	1003 ± 23
49.5 + IN	60	1002 ± 36	1049 ± 53	936 ± 41
0.0	0	589 ± 10	478 ± 10	539 ± 11
0.0	120	1216 ± 129	1164 ± 75	1086 ± 45
IN	120	1181 ± 107	1115 ± 67	1034 ± 22
3.3	120	1048 ± 111	1061 ± 40	1188 ± 119
16.5	120	1028 ± 66	1041 ± 48	998 ± 39
33.3	120	983 ± 50	1089 ± 20	1018 ± 25
49.5	120	1034 ± 26	1063 ± 42	1095 ± 27
49.5 + IN	120	1118 ± 36	1023 ± 31	1004 ± 30

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN) or corticosterone. The values shown are expressed in units per liter ± the standard error of the means of three experiments in duplicate. The exogenous glucose and lactate concentrations were 5 mM and 10 mM, respectively. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.

Table 7. Effects of Insulin, Glucose, Corticosterone and Cortisol on Supernatant Glutamic Oxaloacetic Transaminase Levels in Isolated Hepatocytes Incubated at 42°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		42°C	42°C	Difference
Control	0	386 ± 45* ^{d}	388 ± 69* ^{a}	N. S.
Control	30	633 ± 30 ^{c}	533 ± 98 ^{a}	N. S.
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	553 ± 31 ^{c}	495 ± 96 ^{a}	N. S.
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	531 ± 30 ^{cd}	483 ± 94 ^{a}	N. S.
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	533 ± 33 ^{cd}	481 ± 96 ^{a}	N. S.
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	557 ± 29 ^{c}	464 ± 87 ^{a}	N. S.
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	589 ± 36 ^{c}	496 ± 88 ^{a}	N. S.
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	788 ± 50 ^{b}	542 ± 112 ^{a}	N. S.
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	1177 ± 105 ^{a}	489 ± 54 ^{a}	P < 0.0002
IN (1.0 uU/ml) CL (49.5 ug/ml	30	1232 ± 80 ^{a}	594 ± 72 ^{a}	P < 0.0001

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in units per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

(a, b, c, d) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

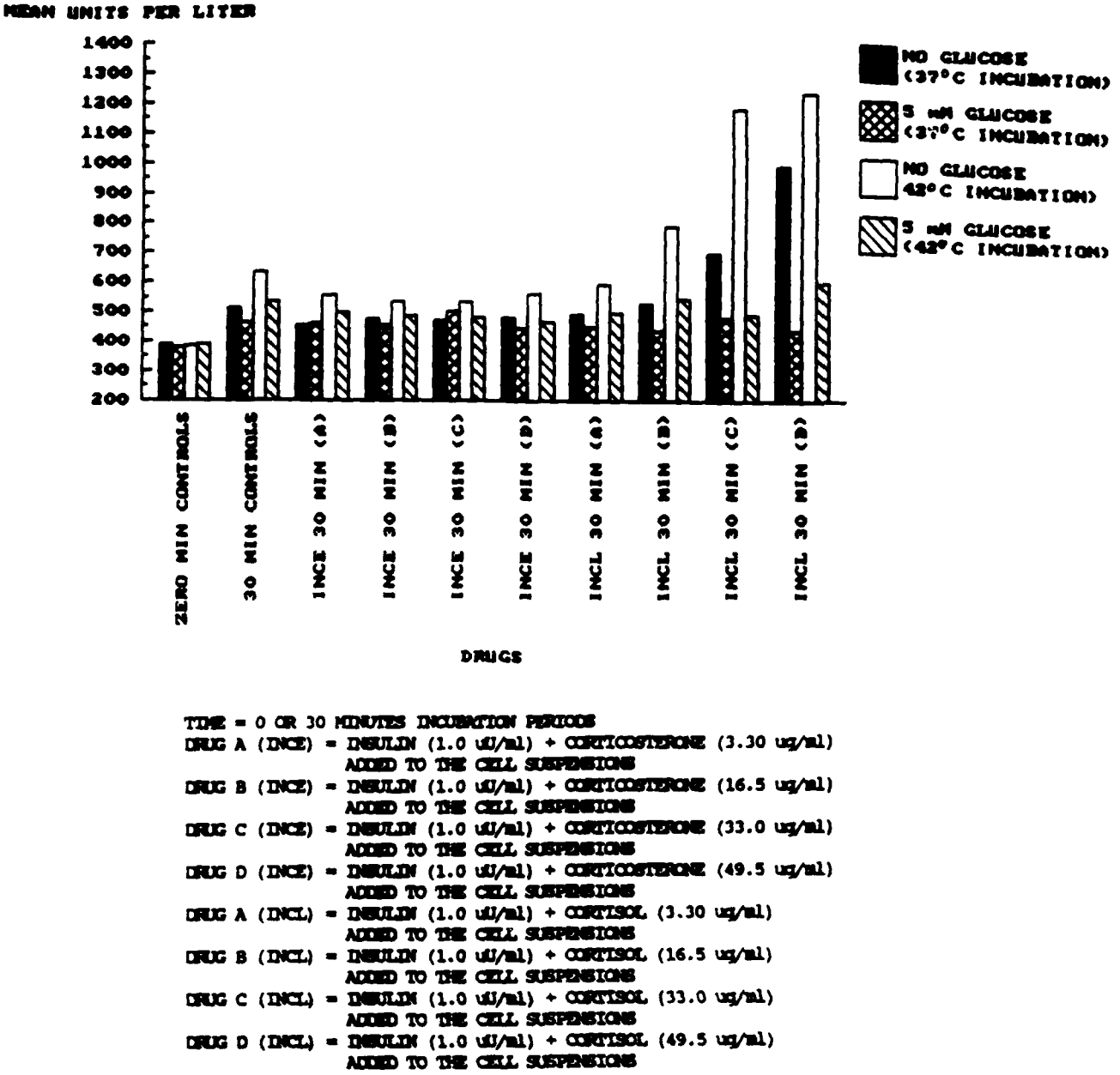
(1) Probability of difference based on t test. N. S., not significant at five percent.

Table 8. Effects of Insulin, Glucose, Corticosterone and Lactate on Supernatant Glutamic Oxaloacetic Transaminase Levels in Isolated Hepatocytes Incubated at 42°C.

Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	584 ± 12	519 ± 10	506 ± 41
0.0	60	1325 ± 52	1331 ± 113	1333 ± 102
IN	60	1454 ± 36	1276 ± 80	1340 ± 116
3.3	60	1314 ± 26	1293 ± 93	1519 ± 128
16.5	60	1319 ± 79	1323 ± 111	1438 ± 109
33.0	60	1414 ± 94	1436 ± 195	1388 ± 190
49.5	60	1515 ± 188	1538 ± 235	1579 ± 237
49.5 + IN	60	1195 ± 150	1595 ± 246	1459 ± 201
0.0	0	584 ± 12	519 ± 10	506 ± 41
0.0	120	1813 ± 116	2143 ± 228	1745 ± 144
IN	120	1684 ± 54	1785 ± 118	1608 ± 118
3.3	120	1627 ± 70	1573 ± 110	1896 ± 74
16.5	120	1610 ± 145	1659 ± 142	1805 ± 236
33.0	120	1673 ± 180	1935 ± 221	1843 ± 215
49.5	120	1979 ± 258	2088 ± 224	1890 ± 192
49.5 + IN	120	2045 ± 96	1929 ± 205	1826 ± 163

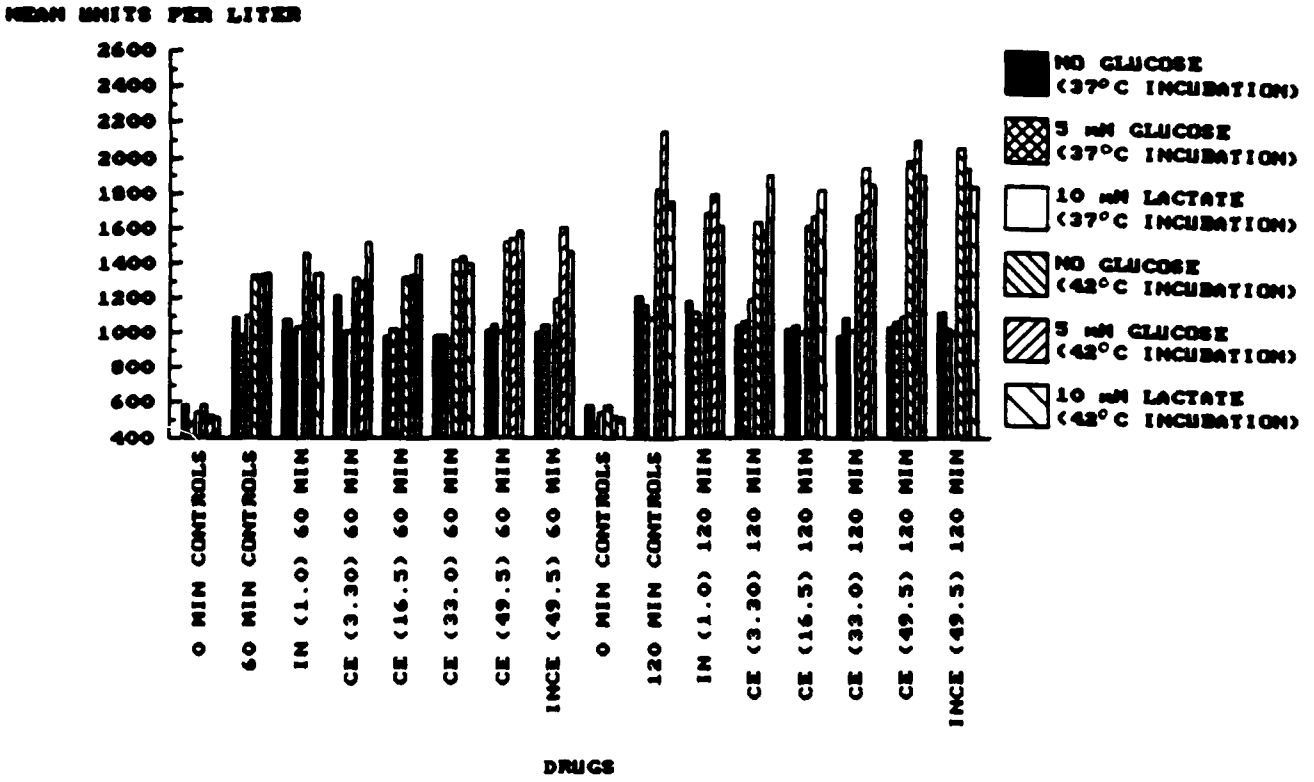
Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN) or corticosterone. The values shown are expressed in units per liter ± the standard error of the means of three experiments in duplicate. The exogenous glucose and lactate concentrations were 5 mM and 10 mM, respectively. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.

(a, b) Any two means within a column with the same superscript are not significant at the five percent level of probability using the LSD test. LSD at 0.05 = 316.3.



TIME = 0 OR 30 MINUTES INCUBATION PERIODS
DRUG A (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG A (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 3. Effects of Various Insulin, Corticosterone and Cortisol Concentrations on Supernatant Glutamic Oxaloacetate Transaminase Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 30 Minutes with 5 mM Glucose or without Glucose.



TIME = 0, 60 OR 120 MINUTES INCUBATION PERIODS
IN = INSULIN (1.0 uU/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
INCE = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 4. Effects of Various Insulin and Corticosterone Concentrations on Supernatant Glutamic Oxaloacetate Transaminase Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0, 60 or 120 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.

Table 9. Effects of Insulin, Glucose, Corticosterone and Cortisol on Sodium Levels in Isolated Hepatocytes Incubated at 37°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		37°C	37°C	Difference
Control	0	152 ± 6*	122 ± 9*	P < 0.02
Control	30	149 ± 7	123 ± 9	P < 0.05
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	148 ± 4	121 ± 9	P < 0.02
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	153 ± 7	123 ± 10	P < 0.04
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	153 ± 7	124 ± 9	P < 0.04
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	163 ± 11	125 ± 10	P < 0.03
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	151 ± 5	122 ± 10	P < 0.03
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	155 ± 7	123 ± 9	P < 0.02
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	157 ± 11	123 ± 9	P < 0.02
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	142 ± 2	126 ± 10	N. S.

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in milliequivalents per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

Means within the columns are not significantly different from each other.

(1) Probability of difference based on t test. N. S., not significant at five percent.

Table 10. Effects of Insulin, Glucose, Corticosterone and Cortisol on Sodium Levels in Isolated Hepatocytes Incubated at 42°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature	
		42°C	42°C	Difference
Control	0	149 ± 5*	126 ± 9*	N. S.
Control	30	145 ± 4	128 ± 9	N. S.
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	149 ± 5	126 ± 9	P < 0.05
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	151 ± 7	124 ± 9	P < 0.04
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	158 ± 10	128 ± 10	P < 0.06
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	162 ± 12	128 ± 9	P < 0.05
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	150 ± 5	123 ± 9	P < 0.03
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	156 ± 8	125 ± 10	P < 0.03
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	143 ± 1	126 ± 9	N. S.
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	158 ± 13	131 ± 10	N. S.

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in milliequivalents per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocytes suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

Means within columns are not significantly different from each other.

(1) Probability of difference based on t test. N. S., not significant at five percent.

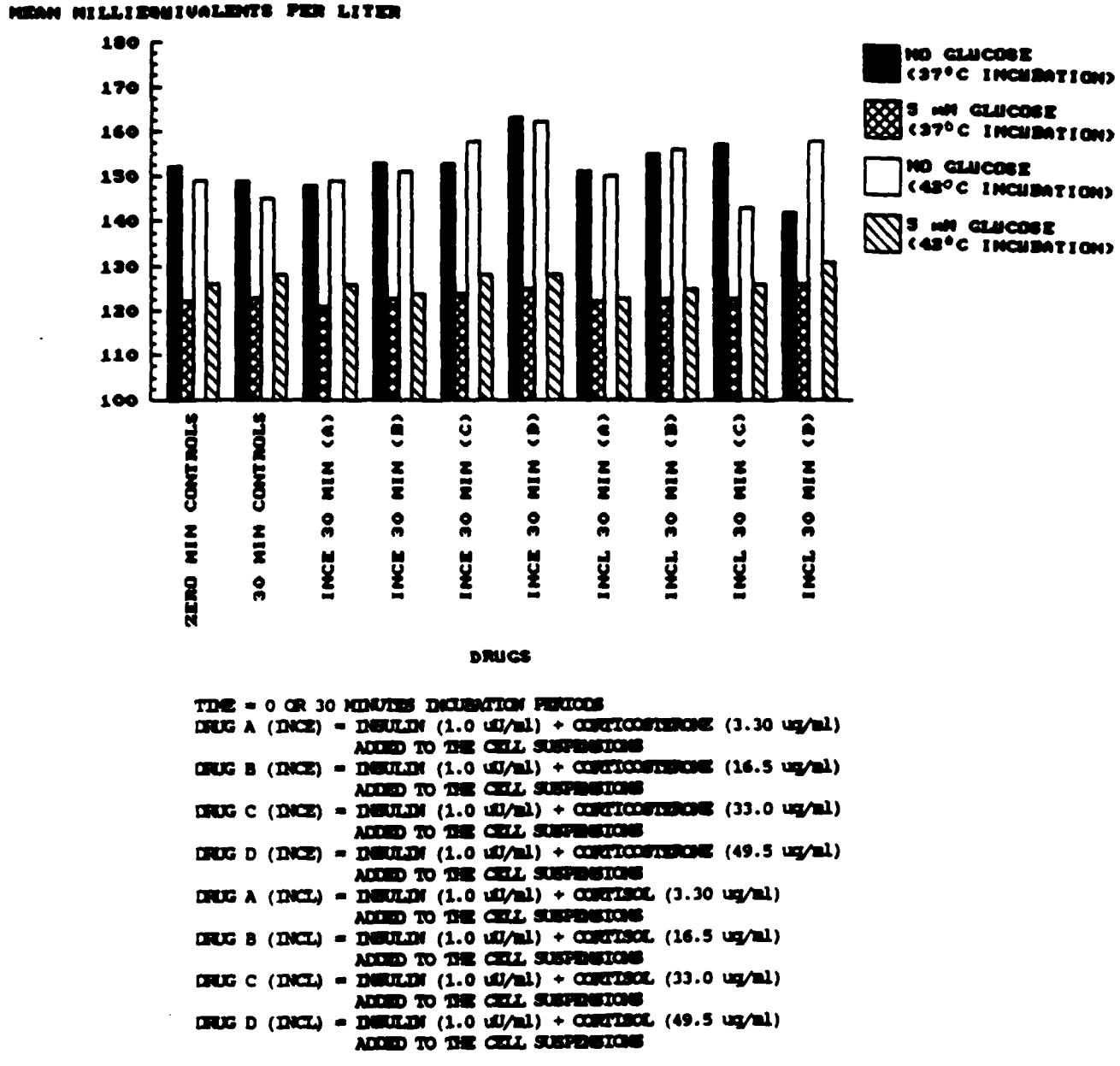


Figure 5. Effects of Various Insulin, Corticosterone and Cortisol Concentrations on Sodium Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 30 Minutes with 5 mM Glucose or without Glucose.

Table 11. Effects of Insulin, Glucose, Corticosterone and Cortisol on Potassium Levels in Isolated Hepatocytes Incubated at 37°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature
		37°C	37°C
Control	0	6.5 ± 0.3*	5.3 ± 0.4*
Control	30	6.4 ± 0.2	5.3 ± 0.4
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	6.2 ± 0.1	5.2 ± 0.5
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	6.4 ± 0.2	5.2 ± 0.5
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	6.2 ± 0.3	5.3 ± 0.5
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	6.6 ± 0.4	5.1 ± 0.6
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	6.3 ± 0.2	5.4 ± 0.4
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	6.5 ± 0.3	5.6 ± 0.4
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	6.5 ± 0.3	5.3 ± 0.4
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	6.8 ± 0.6	5.3 ± 0.4

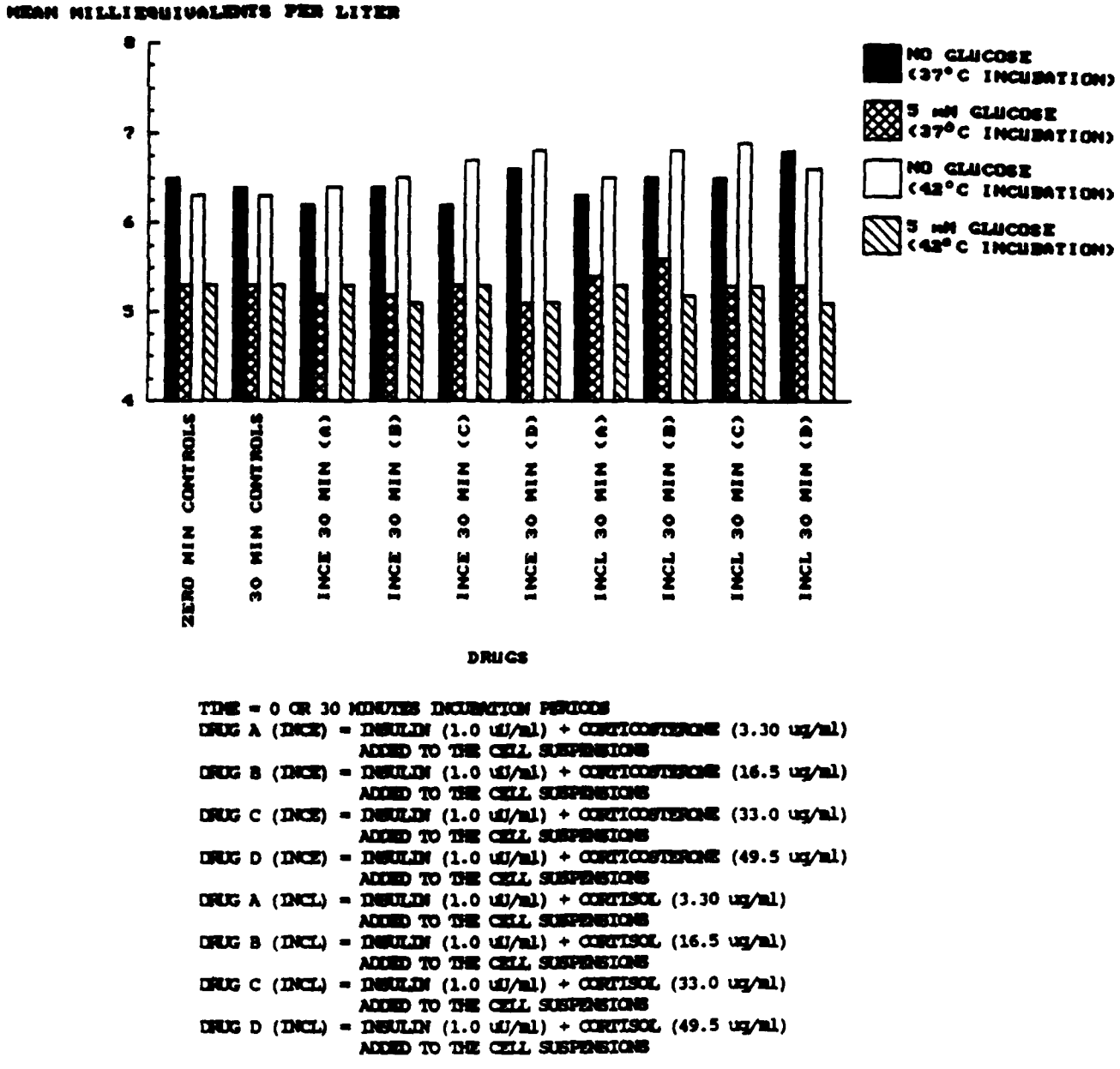
Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in milliequivalents per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

Table 12. Effects of Corticosterone and Insulin on Potassium Levels in Isolated Hepatocytes Incubated at 37°C.

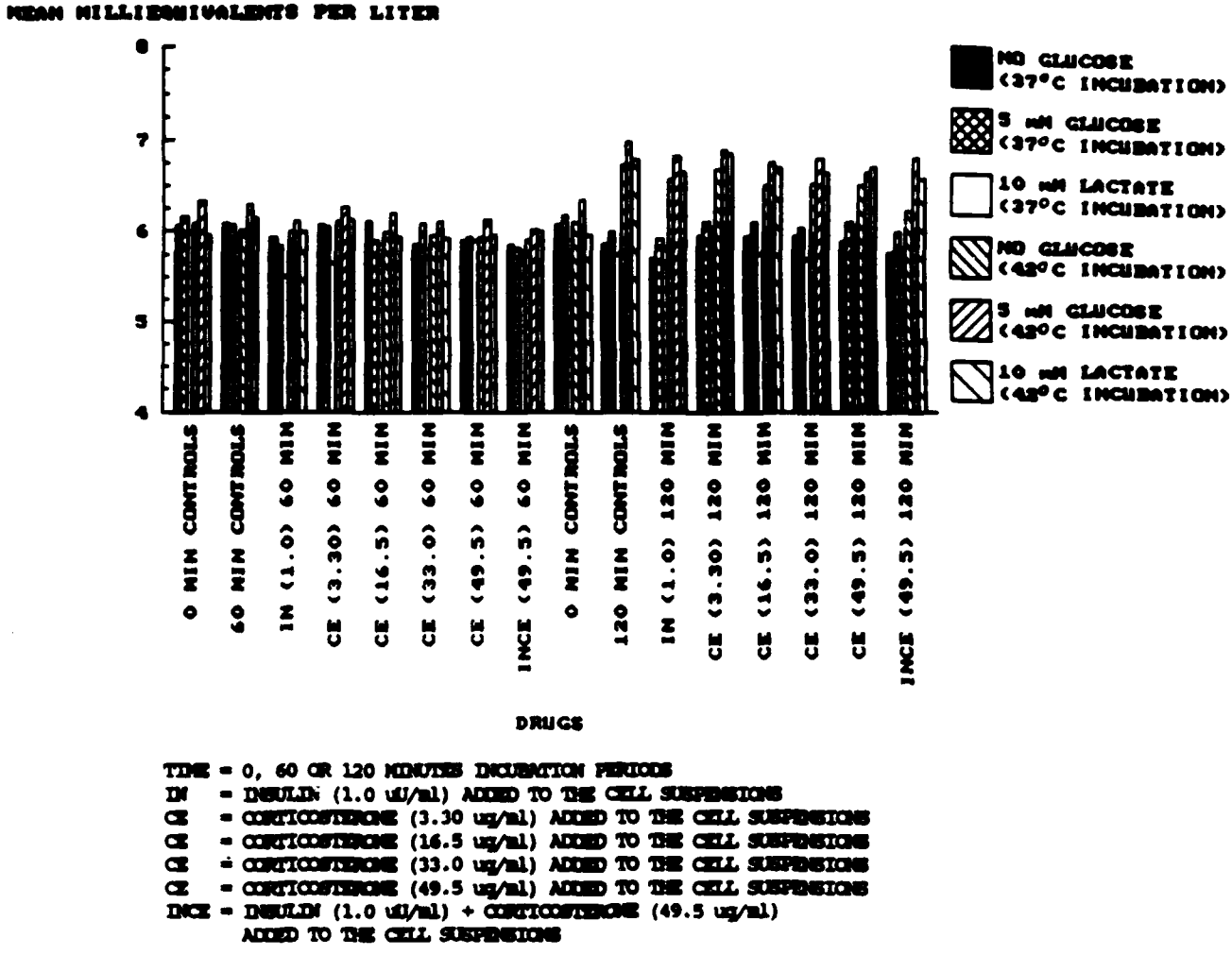
Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	6.06 ± 0.18	6.16 ± 0.15	5.99 ± 0.12
0.0	60	6.09 ± 0.27	6.06 ± 0.16	5.68 ± 0.15
IN	60	5.93 ± 0.03	5.84 ± 0.07	5.49 ± 0.24
3.3	60	6.06 ± 0.31	6.05 ± 0.23	5.64 ± 0.18
16.5	60	6.10 ± 0.05	5.90 ± 0.17	5.73 ± 0.10
33.0	60	5.85 ± 0.19	6.09 ± 0.14	5.73 ± 0.11
49.5	60	5.89 ± 0.30	5.94 ± 0.17	5.90 ± 0.12
49.5 + IN	60	5.84 ± 0.30	5.80 ± 0.12	5.74 ± 0.30
0.0	0	6.06 ± 0.18	6.16 ± 0.15	5.99 ± 0.12
0.0	120	5.85 ± 0.26	5.98 ± 0.14	5.75 ± 0.20
IN	120	5.70 ± 0.24	5.91 ± 0.17	5.81 ± 0.26
3.3	120	5.95 ± 0.26	6.10 ± 0.17	5.79 ± 0.21
16.5	120	5.95 ± 0.25	6.10 ± 0.19	5.76 ± 0.17
33.0	120	5.96 ± 0.25	6.04 ± 0.16	5.71 ± 0.15
49.5	120	5.90 ± 0.25	6.10 ± 0.13	5.78 ± 0.14
49.5 + IN	120	5.76 ± 0.23	5.98 ± 0.14	5.79 ± 0.12

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 37°C with and without corticosterone for the time periods indicated. These cells were incubated in the presence or absence of glucose (5 mM) or lactate (10 mM). The values were obtained by the analyses of the supernatants from the cells suspensions and are expressed as milliequivalents per liter ± standard error of the means of three experiments in duplicate. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.



TIME = 0 OR 30 MINUTES INCUBATION PERIODS
DRUG A (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG A (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 6. Effects of Various Insulin, Corticosterone and Cortisol Concentrations on Potassium Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 30 Minutes with 5 mM Glucose or without Glucose.



TIME = 0, 60 OR 120 MINUTES INCUBATION PERIODS
IN = INSULIN (1.0 uU/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
INCE = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 7. Effects of Various Insulin and Corticosterone Concentrations on Potassium Released from Isolated Hepatocytes Incubated at 37°C or 42°C for 0, 60 or 120 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.

Table 13. Effects of Insulin, Glucose, Corticosterone and Cortisol on Potassium Levels in Isolated Hepatocytes Incubated at 42°C

Drug	Incubation Time (min)	No Glucose Incubation Temperature	5 mM Glucose Incubation Temperature
		42°C	42°C
Control	0	6.3 ± 0.2*	5.3 ± 0.3*
Control	30	6.3 ± 0.2	5.3 ± 0.4
IN (1.0 uU/ml) CE (3.30 ug/ml)	30	6.4 ± 0.2	5.3 ± 0.4
IN (1.0 uU/ml) CE (16.5 ug/ml)	30	6.5 ± 0.3	5.1 ± 0.4
IN (1.0 uU/ml) CE (33.0 ug/ml)	30	6.7 ± 0.4	5.3 ± 0.4
IN (1.0 uU/ml) CE (49.5 ug/ml)	30	6.8 ± 0.4	5.1 ± 0.4
IN (1.0 uU/ml) CL (3.30 ug/ml)	30	6.5 ± 0.2	5.3 ± 0.3
IN (1.0 uU/ml) CL (16.5 ug/ml)	30	6.8 ± 0.4	5.2 ± 0.4
IN (1.0 uU/ml) CL (33.0 ug/ml)	30	6.9 ± 0.5	5.3 ± 0.4
IN (1.0 uU/ml) CL (49.5 ug/ml)	30	6.6 ± 0.6	5.1 ± 0.4

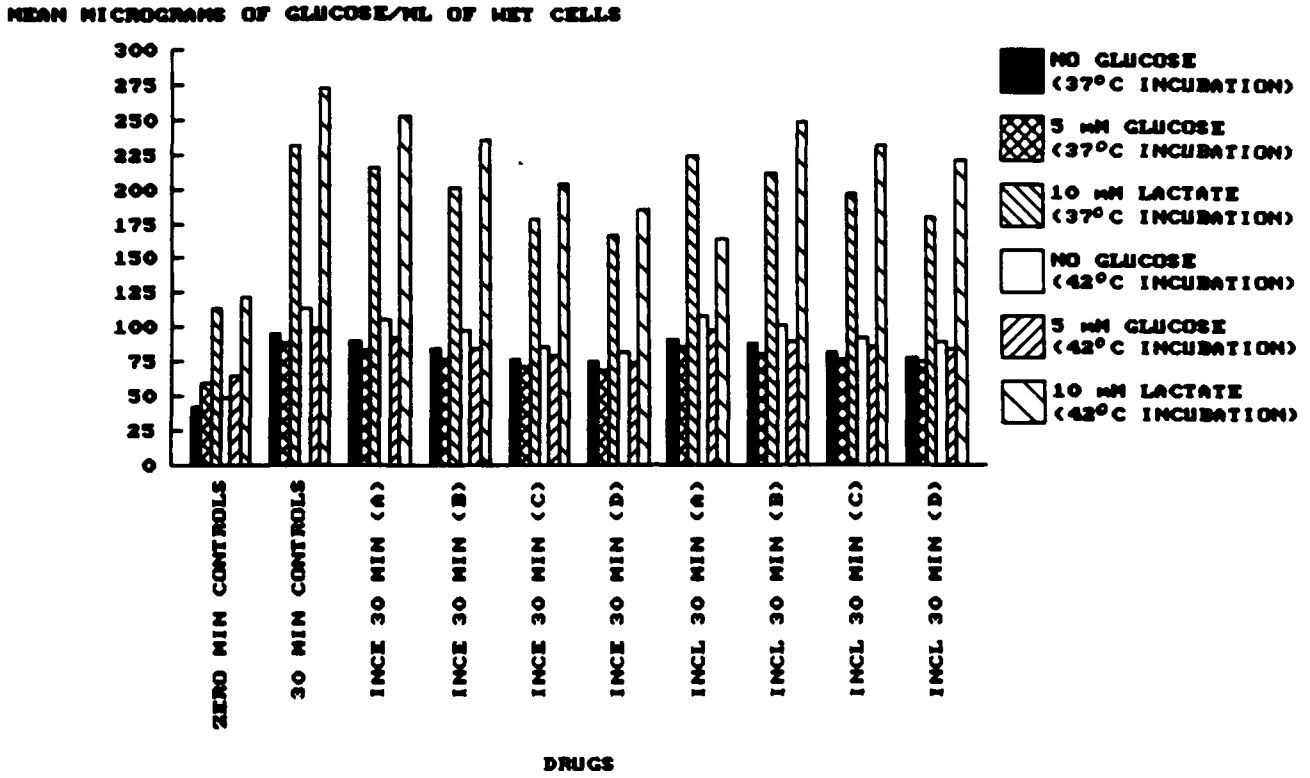
Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without insulin (IN), corticosterone (CE) and cortisol (CL) added, as noted above. The values are the means in milliequivalents per liter ± the standard error of the means of 6 determinations on the supernatants obtained from the hepatocyte suspensions (in which the initial cells were either resuspended without glucose or in 5 mM glucose) after each drug treatment.

(*) The initial cell suspensions were chilled in an ice/water bath and the supernatants were obtained without any incubation.

Table 14. Effects of Corticosterone and Insulin on Potassium Levels in Isolated Hepatocytes Incubated at 42°C.

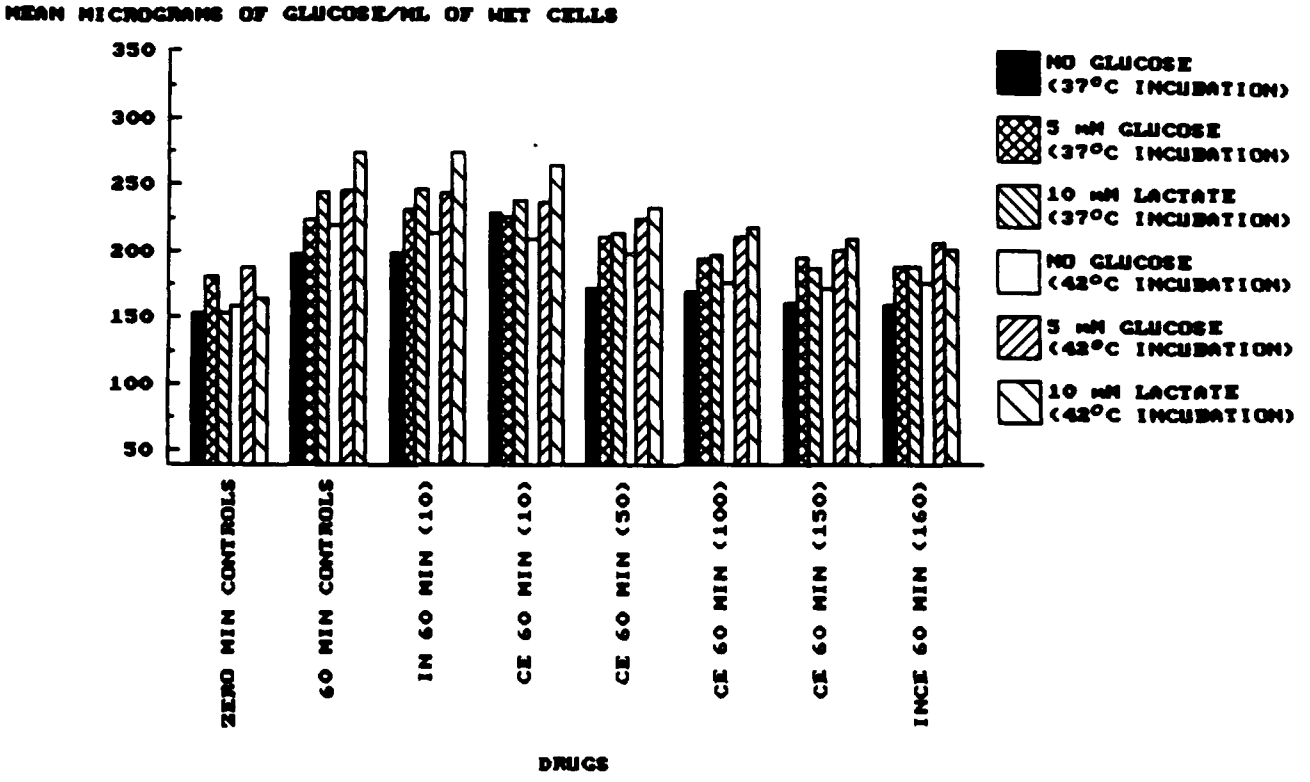
Corticosterone ug/ml	Incubation Time (min)	None	Glucose	Lactate
0.0	0	6.08 ± 0.17	6.34 ± 0.23	5.96 ± 0.13
0.0	60	6.00 ± 0.26	6.29 ± 0.06	6.14 ± 0.12
IN	60	5.98 ± 0.30	6.10 ± 0.21	5.99 ± 0.15
3.3	60	6.10 ± 0.25	6.25 ± 0.17	6.13 ± 0.15
16.5	60	5.98 ± 0.20	6.19 ± 0.14	5.94 ± 0.14
33.0	60	5.95 ± 0.17	6.11 ± 0.10	5.91 ± 0.11
49.5	60	5.94 ± 0.15	6.13 ± 0.10	5.96 ± 0.10
49.5 + IN	60	5.90 ± 0.23	6.00 ± 0.12	5.99 ± 0.17
0.0	0	6.08 ± 0.17	6.34 ± 0.23	5.96 ± 0.13
0.0	120	6.71 ± 0.41	6.96 ± 0.34	6.76 ± 0.30
IN	120	6.56 ± 0.37	6.81 ± 0.34	6.64 ± 0.27
3.3	120	6.68 ± 0.38	6.88 ± 0.34	6.84 ± 0.28
16.5	120	6.51 ± 0.33	6.75 ± 0.27	6.70 ± 0.24
33.0	120	6.53 ± 0.38	6.79 ± 0.30	6.64 ± 0.22
49.5	120	6.50 ± 0.39	6.64 ± 0.31	6.69 ± 0.20
49.5 + IN	120	6.21 ± 0.33	6.78 ± 0.42	6.55 ± 0.19

Isolated hepatic parenchymal cells (50 mg/ml) from 450 to 550 gram male Sprague Dawley rats were incubated at 42°C with or without corticosterone for the periods indicated. These cells were incubated in the presence or absence of glucose (5 mM) or lactate (10 mM). The values were obtained by the analyses of the supernatants from the cell suspensions and are expressed as milliequivalents per liter ± standard error of the means of three experiments in duplicate. In cases where insulin (IN) was added, its concentration was 1.0 uU/ml.



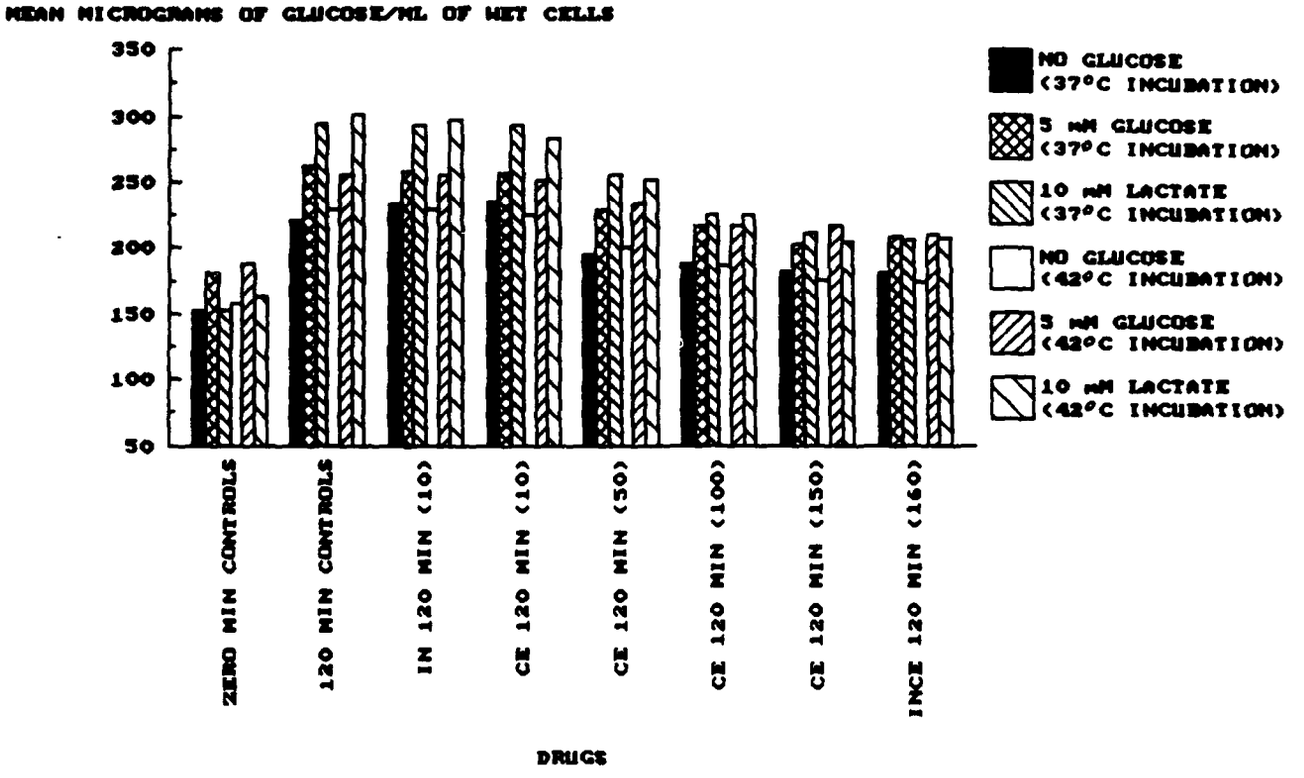
TIME = 0 OR 30 MINUTES INCUBATION PERIODS
DRUG A (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCE) = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG A (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (3.30 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG B (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (16.5 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG C (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (33.0 ug/ml) ADDED TO THE CELL SUSPENSIONS
DRUG D (INCL) = INSULIN (1.0 uU/ml) + CORTISOL (49.5 ug/ml) ADDED TO THE CELL SUSPENSIONS

Figure 8. Effects of Various Insulin, Corticosterone and Cortisol Concentrations on Glucose Production from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 30 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.



TIME = 0 OR 60 MINUTES INCUBATION PERIODS
IN = INSULIN (1.0 uU/ml) 10 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 10 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 50 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 100 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 150 ul ADDED TO THE CELL SUSPENSIONS
INCE = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml)
(10 ul OF IN + 150 ul OF CE ADDED TO THE CELL SUSPENSIONS)

Figure 9. Effects of Various Insulin and Corticosterone Concentrations on Glucose Production from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 60 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.



TIME = 0 OR 120 MINUTES INCUBATION PERIODS
IN = INSULIN (1.0 uU/ml) 10 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 10 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 50 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 100 ul ADDED TO THE CELL SUSPENSIONS
CE = CORTICOSTERONE (49.5 ug/ml) 150 ul ADDED TO THE CELL SUSPENSIONS
INCE = INSULIN (1.0 uU/ml) + CORTICOSTERONE (49.5 ug/ml)
(10 ul OF IN + 150 ul OF CE ADDED TO THE CELL SUSPENSIONS)

Figure 10. Effects of Various Insulin and Corticosterone Concentrations on Glucose Production from Isolated Hepatocytes Incubated at 37°C or 42°C for 0 or 120 Minutes with 5 mM Glucose, 10 mM Lactate or without Glucose.

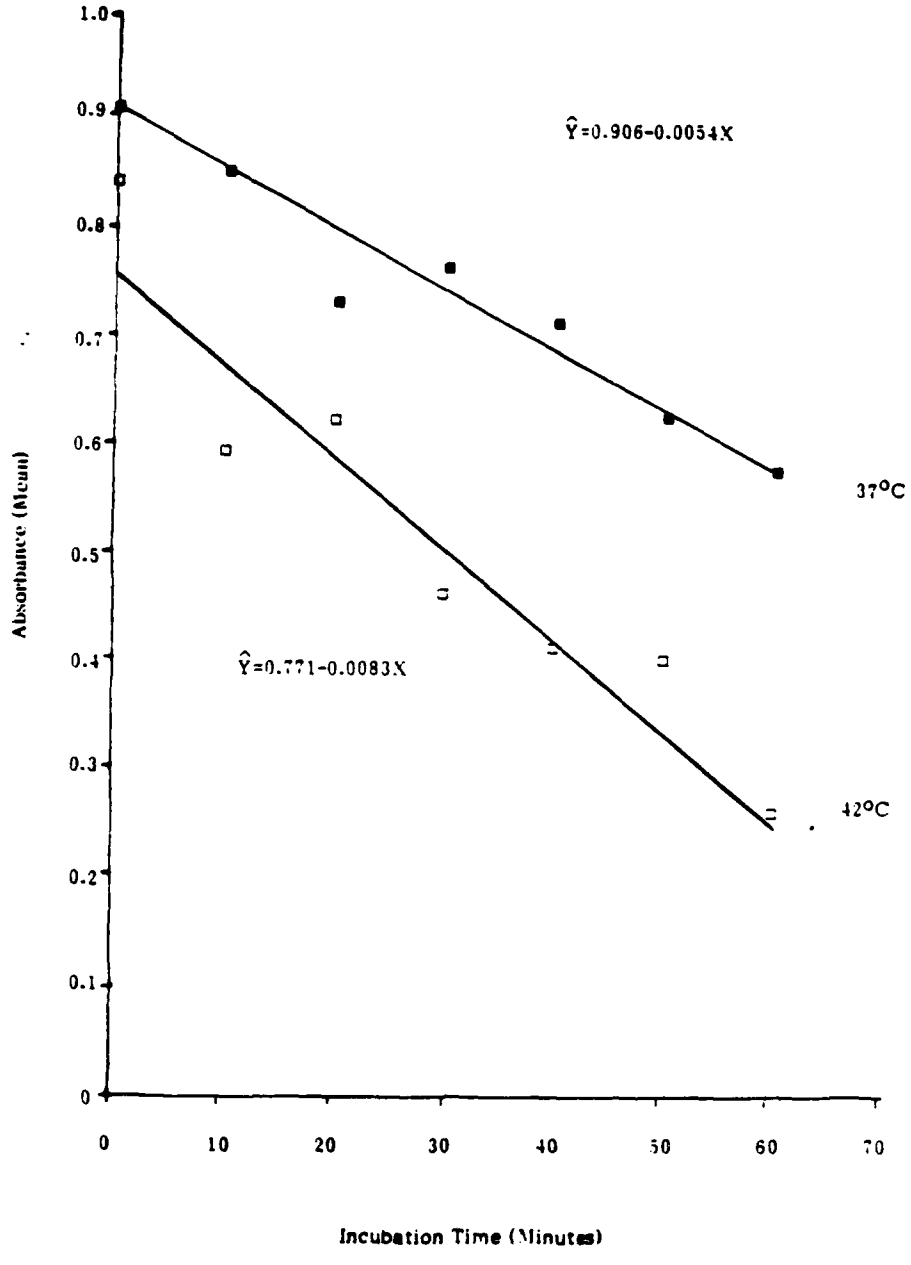


Figure 11. Linear Regression Plot of Glycogen Breakdown in Rat Liver Cells.

B. DETERMINATION OF CORTICOSTERONE AND SERUM PROFILES IN HEATED VERSUS CONTROL RATS

THE SETTING

The Carver Research Foundation of Tuskegee University team conducted this aspect of the project in December 1986 at USARIEM in Natick, Massachusetts.

EXPERIMENTAL METHODS

Male Sprague Dawley rats, obtained from Charles River Breeding Lab, were heated to 42.2°C - 42.6°C. These animals were then removed from the heating chamber and monitored until their rectal temperatures dropped to or below 40.4°C. Blood samples from each animal were obtained by cardiac puncture when the rectal temperature dropped to or below 40.4°C. Blood samples were prepared for analyses of the sera.

The serum samples were analyzed in duplicate for corticosterone with the aid of a radioimmunoassay (31, 33) kit obtained from Cambridge Medical Diagnostics. Each serum sample was also analyzed for glucose, K+, SGOT, LDH and CPK levels.

Unheated rats were used as controls.

Hematocrits were done for each of the rats.

RESULTS

Hematocrits for the heat-stressed rats averaged 50 $\pm$ 3%, whereas those for the unheated control animals averaged 48 $\pm$ 1%. However, there was no significant difference in the hematocrits obtained.

In a radioactive evaluation using a I-125 labeled corticosterone MSA RIA kit it was determined that the control rat sera contained 44 $\pm$ 2 nanograms of endogenous corticosterone per milliliter of blood serum. The heat-stressed animals demonstrated a rise in the radioactive corticosterone content to 47 $\pm$ 2 nanograms per milliliter of blood serum within the first hour after the heating regimens of the animals were discontinued.

The blood serum of the heat-stressed animals was also shown to contain approximately two-fold more LDH, SGOT and CPK (Table 15, Figure 12) as compared to the control animals and was also shown to be significantly different ($p < 0.007$, $p < 0.001$ and $p < 0.02$, respectively) for the LDH, SGOT and CPK serum levels. The blood serum from these enzyme determinations were obtained at the same time as that for the corticosterone MSA RIA studies. The potassium (K+) levels were increased by approximately 1/2 milliequivalents/liter for the heat-stressed animals, but were not significantly different when compared to the control animals.

Table 15. Serum Profile of Heat-Stressed Versus Control Rats

	K+	LDH	SGOT	CPK
Control	5.3 ± 0.2	842 ± 55	128 ± 7	2525 ± 220
Heat-Stressed	5.8 ± 0.5	1449 ± 180	208 ± 18	4165 ± 579
Difference	N. S.	P < 0.007	P < 0.001	P < 0.02

Eight male Sprague Dawley Rats (450 to 550 grams) were heated (42.2°C - 42.6°C) and removed from the heating chamber. Blood samples were taken and the serum analyzed for enzyme, potassium and hematocrit determinations when their rectal core temperatures dropped to or below 40.4°C. Blood samples of unheated control rats were taken during the same period.

K+ values are expressed as the mean units per liter ± the standard error of the means.

The enzyme values (LDH, SGOT and CPK) are expressed as the mean units per liter ± the standard error of the means.

(1) Probability of difference based on t test. N. S., not significant at five percent.

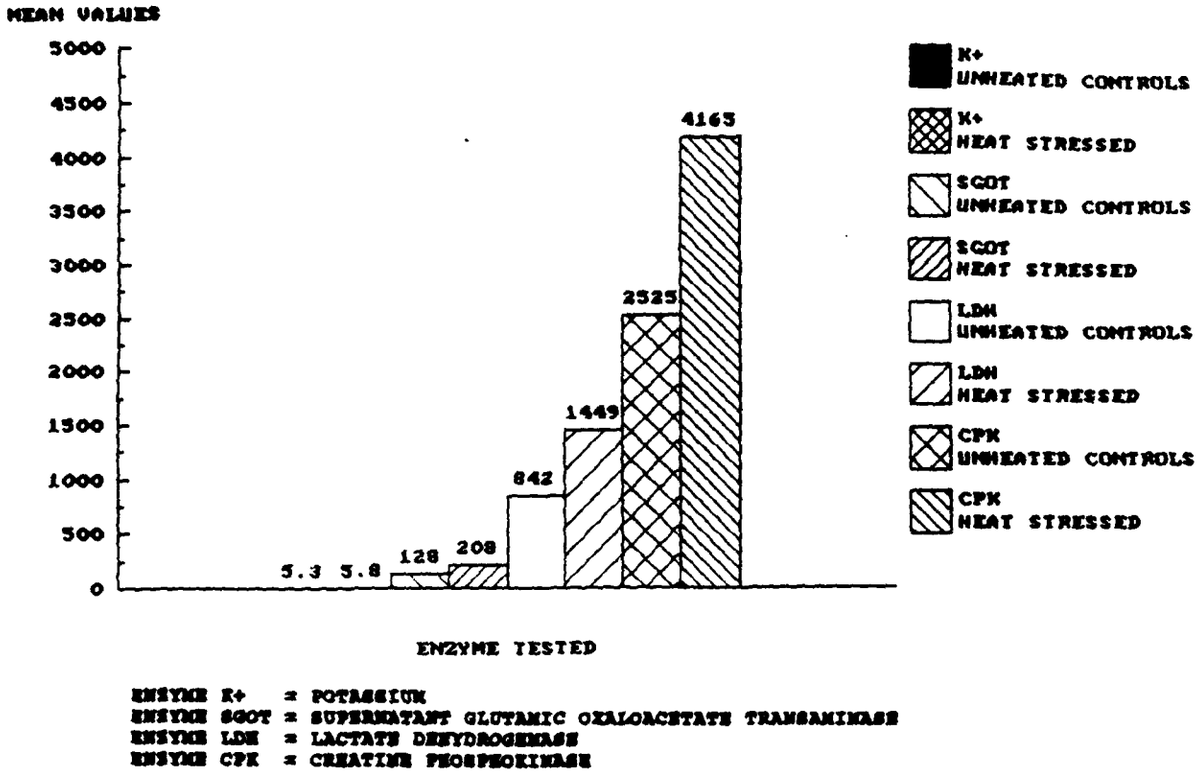


Figure 12. Comparison of the Mean Values of Potassium, Supernatant Glutamic Oxaloacetate Transaminase, Lactate Dehydrogenase and Creatine Phosphokinase Levels in Heat-Stressed or Unheated Animals.

C. CORTICOSTERONE, INSULIN AND GLUCOSE AS A POST-TREATMENT THERAPEUTIC REGIMEN

THE SETTING

The Carver Research Foundation of Tuskegee University team traveled to USARIEM in Natick, Massachusetts, in December 1986, to conduct research required for this aspect of the project.

EXPERIMENTAL METHODS

Some aspects of the experimental methods for this portion of the project appear in Appendix 3 (Attachments 1 and 2). Forty male Sprague Dawley rats (450 to 550 grams) from Charles River Breeding Laboratory were permitted to acclimatize (28°C, 30% relative humidity) for a minimum of 72 hours. The animals received food and water ad libitum before and after heating. On the day of experimentation and prior to the initial heating, each rat was weighed and placed into a restrainer cage. Rectal probes were inserted into each animal and the animals were heated in the heating chamber (Appendix 3, Attachment 1) from 42.2°C to 42.6°C. The animals were removed from the heating chamber after completion of each heating protocol and their rectal temperatures were continually monitored. When their rectal temperatures dropped below 40.4°C, the animal were anesthetized with sodium pentobarbital (i.p.). Twenty of the heat-treated animals received a 0.5 ml intravenous (i.v.) injection of corticosterone (4.95 ug/ml), and 0.5 ml (i.v.) of a mixture of insulin (0.1 uU/ml) and glucose (0.5 mM) via the ascending path of the femoral vein. All of the drugs were diluted in sterile physiological saline for injection. The corticosterone was dissolved in propylene glycol and diluted with saline to make a final propylene glycol concentration of 20%. The remaining twenty animals, which had also been heated in the chamber, received a single i.v. injection of a mixture of sterile physiological saline and propylene glycol which was equal to the combined volumes received by the drug treated animals. The controls and the drug treated animals were placed in their former cages for further monitoring over a twenty-four hour period to ascertain the twenty-four hour survival rate of each of the heat-treated rats.

Blood samples were taken from the survivors via cardiac puncture (vacutainer fitted with a 20 gauge needle). Blood serum was prepared from each sample and analyzed for CPK, glucose, K+, LDH and SGOT levels (Appendix 3, Attachment 2).

Corticosterone (crystalline) was purchased from Sigma. Propylene glycol was purchased from Pitman-Moore Pharmaceutical Company, Atlanta, GA.

RESULTS

The major differences in this series of experiments and preliminary ones are the use of corticosterone that was not attached to BSA, the dissolution of corticosterone in propylene glycol, the intravenous injection of the drug mixtures and the determination of hematocrits.

Control rat weights averaged 450 grams; whereas the rats receiving post-treatments of glucose, corticosterone and insulin averaged 520 grams. The temperatures at which heating was discontinued were 42.4 $\pm$ 0.04°C and 42.3 $\pm$ 0.05°C for controls and post-treated rats, respectively. Their total areas, which are the degree-minutes that they remained above 40.4°C, were not significantly different (Table 16). Potassium and sodium levels were not reduced by the drug treatment mixtures. Perhaps the exogenous insulin concentration was too low. Lactate dehydrogenase (LDH) and serum glutamic oxaloacetate transaminase (SGOT) levels were variable, but were higher for controls than for the post-treated rats. However, glucose and creatine phosphokinase (CPK) values for the control versus post-treated rats were not significantly different (Table 16).

Although the serum profiles did not show any differences in the potassium levels in post-treated versus control rat serum, 15% of the control rats died before the 24-hour survival test period was over, while only 5% treated with the drug mixtures died. The serum profiles of the animals that died are not included among these data. Details on those rats that died are given in Table 17. When one focuses on the total area as an indicator of survival capability, it is anticipated that rats with total areas above 50 degree-minutes would not survive. Our data show that only one of the four rats whose total area was above 50 degree-minutes did not survive the heat exposure (of course the limited number of experiments which were conducted make these results inconclusive). The other three non-survivors had total areas ranging from 24.99 to 47.7 degree-minutes (Table 17). In an evaluation of the results on all 40 of the rats used in this part of the study there were fewer animals whose total area rose above 50 degree-minutes than animals whose total area ranged from 30 to 40 degree-minutes. However, on a percentage basis, a larger percentage (15% of the 20 controls or 10% of the rats with areas above 50 degree-minutes) of the controls in the former group did not survive (Table 18).

Table 16. Serum Profiles of Post-Treated Versus Control Rats

	Temperature (°C) RT	Temperature (°C) Max	Total Area (Deg.-Min.)	K+ MEQ/L	Na+ MEQ/L
Control	42.4±0.04	42.6±0.04	38±1	5.82±0.23	142±0.3
Post-Treated	42.3±0.05	42.4±0.06	36±1	6.04±0.22	143±0.4

	LDH U/L	SGOT U/L	GLU MG/L	CPK U/L
Control	2963±2221	1053±336	164±6	1865±336
Post-Treated	703± 65	779±174	168±4	2008±297

Rats were exposed to heat as indicated in the "Experimental Methods" section. Twenty served as controls and twenty received the post-treatment of glucose, insulin and corticosterone. The values are the means ± standard error of the means for twenty rats in each case. For serum determinations of K+, Na+, LDH, SGOT, GLU and CPK the assays were done in duplicate. Therefore, each value represents the means of forty determinations. Max. = maximum, K+ = potassium, Na+ = sodium, LDH = lactate dehydrogenase, SGOT = serum glutamic oxaloacetate transaminase, GLU = glucose, CPK = creatine phosphokinase, MEQ/L = milliequivalents per liter, U/L = units per liter and MG/L = milligrams per liter. RT = temperature at which each rat was removed from the heating chamber.

Table 17. Individual Heating Profiles of Rats Which Did Not Survive 24-Hours After Heating

	Weight (grams)	Temperature (°C) RT	MAX	Total Area (Deg. - Min.)
Controls				
	515	42.5	42.9	52.3
	501	42.4	42.5	47.7
	488	42.5	42.6	32.9
Post-Treated				
	456	42.5	42.5	24.9

Rats were heated to the temperatures indicated and removed from the chamber. Their temperatures rose slightly, thereafter. Controls were injected (i.v.) with saline containing propylene glycol, while the post-treated rats were injected (i.v.) with 0.5 ml of a mixture of glucose and insulin and 0.5 ml of corticosterone in saline and propylene glycol. All of the above rats were a part of the 40 rats reported in Table 16. Four rats did not survive 24 hours after being heated. RT = temperature at which each rat was removed from the heating chamber; MAX = maximum temperature reached after removal from the heating chamber.

Table 18. Survival of Heat-Stressed Rats Versus Total Area

Total Area (Deg.- Min.)	Total Number of Rats	Number of Rats That Died
Below 40	26	1C, 1T
40 and Above	14	2C

Rats were heated to temperatures ranging from 42°C to 42.9°C. After removal from the heating chamber, their rectal temperatures and total areas were monitored. The number of control rats (C) and the number of post-treated rats (T) that did not survive for 24 hours are indicated.

D. Kinetics of Hormone Uptake in Normal and Hyperthermic Rats

THE SETTING

The research team consisting of Dr. M. E. M. Tolbert, Dr. A. Weaver, Mrs. V. E. Hicks and Ms. C. E. Belton of the Carver Research Foundation of Tuskegee University traveled to USARIEM in Natick, MA, in order to conduct the remaining portion of the kinetic studies. This portion of the project was completed in December, 1986.

EXPERIMENTAL METHODS

Kinetic studies were conducted using I-125 labeled corticosterone. Male Sprague Dawley rats (CD, Strain, Charles River Breeding Laboratory) weighing 450 to 550 grams were allowed to acclimatize for a minimum of 72 hours at 28°C and 30% relative humidity. Twenty four of the forty eight rats used in this phase of the investigations were used as the unheated controls. The remaining animals were subjected (as described in Appendix 3, Attachment 1) to temperatures ranging from 42.0°C to 42.6°C. These rats were removed from the heating chamber and allowed to cool until their rectal core temperatures dropped to or below 40.4°C. Each rat was anesthetized with sodium pentobarbital (i.p.) and approximately 4 microcuries of the radiolabeled compound in saline was then injected via the ascending path of the femoral vein. The rats were sacrified for blood, urine and organ samples (i.e., liver, heart, kidneys, adrenals, brain, femur, lungs, muscle, pituitary, small intestines, spleen and testes) in order to assay for the body distribution patterns of the radiolabeled compound on a time course basis. Note that drug injections, except sodium pentobarbital, were made intravenously.

I-125 labeled corticosterone was purchased from Cambridge Medical Diagnostics, Inc., Billerica, MA.

RESULTS

The results of the biodistribution studies of iodine-125 labeled corticosterone were determined in either heat-stressed or control male rats. The heat-stressed or control rats were divided into groups of three animals per time period and were sacrificed at time intervals of 0.25, 0.50, 1, 2, 5, 15, 30 or 60 minutes after the i.v. injection of 3.8 to 4.2 uCi of the radiolabeled compound. The results are shown in Tables 19 - 22, Figures 13 - 36.

Iodine-125 labeled corticosterone cleared rapidly from the blood of both the heat-stressed and control animals with 0.171 $\pm$ 0.012 to 0.061 $\pm$ 0.002% or 0.146 $\pm$ 0.007 to 0.045 $\pm$ 0.000 %kg-dose/gram, respectively, of the radiolabeled compound remaining in the circulation at 0.25 to 2 minutes after injection (Tables 19, 21, Figures 13 - 18, 20, 22, 23, 26, 31, 36). There was a significantly ($p < 0.04$) greater uptake and retention of the radiolabeled compound in the heat-stressed animals at the 0.25 to 2 minute time period. After two minutes, the blood levels for the heat-stressed animals had dropped to 0.032 $\pm$ 0.003%, while the kidney and urine levels had risen to 0.141 $\pm$ 0.015 and 0.085 $\pm$0.014%, respectively, at the 5 minute post-injection time period (Tables

19 - 20 , Figures 13 - 18, 20, 22, 23, 26, 31, 36). In the control animals, the urine and kidneys demonstrated varying distribution patterns (Tables 21 - 22, Figures 14 - 18, 23) and were significantly ($p < 0.01$) different at the longer time periods in the control rats. However, the heat-stressed and control animal kidney localization patterns were similar throughout the experimentation (Tables 19 - 22, Figures 14 - 18, 23).

Varying distribution patterns of the blood (Figures 13, 16, 17, 20, 22, 23, 26, 31, 36) were exhibited. There was a significant ($p < 0.02 - p < 0.04$) decrease in the blood retention of the radiolabeled compound at the 0.25 to 60 minute time periods in both the heat-stressed or control rats. However, there was a higher uptake and longer retention time of the radiolabeled compound in the blood of the heat-stressed rats.

There was a rapid decrease with time in the heart distribution patterns in the heat-stressed animals as compared to the control animals (Tables 19 - 22, Figures 19 - 20). The heart localization patterns of the control animals peaked after 0.50 to 1 minute of exposure. With the exception of these two periods, the heart localization patterns of the control animals and heat-stressed animals were not significantly different (Tables 20 - 22, Figures 19 - 20).

There was a significant ($p < 0.03 - p < 0.04$) increase in the liver distribution pattern noted in the heat-stressed and control animals which peaked at the 2 or 5 minute post-injection time period for the heat-stressed or control animals, respectively. This increase in the liver uptake and retention of the radiolabeled compound was more defined in the heat-stressed rats as compared to the unheated controls. However, the livers from the heat-stressed animals seem to have a faster and longer retention time of the radiolabeled compound as well as to demonstrate a biphasic uptake at the 2 and 60 minute post-injection time periods (Tables 19 - 22, Figures 20 - 23). Further analysis of the liver uptake of I-125 labeled corticosterone was done in which the liver subcellular localization patterns of the labeled compound were analyzed. The liver subcellular fraction study is addressed in the next section.

Rat lungs demonstrated a decreasing distribution pattern for both experimental groups. However, the lungs in the heat-stressed animals seem to have a higher retention of the radiolabeled compound, with this retention being biphasic at the 0.25 and 60 minute post-injection time periods, respectively (Tables 19 - 22, Figures 20, 24).

The spleen of the control animals demonstrated a relatively stable radiolabel distribution pattern. However, there was a significantly ($p < 0.05$) greater uptake of the radiolabeled compound in the heat-stressed animals at the 60 minute post-injection time period (Tables 19 - 22, Figures 23, 25 - 26).

The adrenal distribution patterns of the heat-stressed animals were significantly ($p < 0.05$) higher than the control animals at the 0.25 and 60 minute post-injection time periods, but were variable for the other test periods in both groups of animals (Tables 19 - 22, Figures 18, 27).

The pituitary and brain localization patterns were similar throughout these investigations. However, the heat-stressed animals seem to have a higher uptake and longer retention time of the radiolabeled compound (Tables 19 - 22, Figures 28 - 31).

Of the other tissues and organs tested (i.e., femur, muscle, small intestines and testes), the small intestines and muscle demonstrated increasing localization patterns, while the uptake in the femur was unchanged and the localization patterns in the testes were variable in both groups tested (Tables 19 - 22, Figures 26, 32 - 36).

After 60 minutes, there might be a significant variation in the uptake and retention patterns of the radiolabeled compound in the control and heat-stressed organs and tissue samples. However, investigations were not done beyond the 60 minute time period. With the exception of the high blood perfusion and blood containing organs (i.e., heart and femur), the levels of the radiolabeled compound were higher in the heat-stressed rats than in the control animals.

Table 19. Tissue Distribution of Iodine-125 Labeled Corticosterone in Heat-Stressed Male Rats Exposed From 0.25 to 2 Minutes

Tissue	Time Period in Minutes 0.25	0.50	1.00	2.00	L.S.D. at 0.05
Adrenals	0.147 ± 0.067	0.060 ± 0.041	0.142 ± 0.022	0.094 ± 0.011	
Blood	0.171 ± 0.012[a]	0.154 ± 0.018[ab]	0.106 ± 0.004[b]	0.061 ± 0.002[c]	P < 0.038
Brain	0.006 ± 0.001	0.003 ± 0.001	0.003 ± 0.000	0.002 ± 0.000	
Femur	0.007 ± 0.000	0.007 ± 0.002	0.010 ± 0.001	0.010 ± 0.001	
Heart	0.068 ± 0.012	0.041 ± 0.017	0.042 ± 0.002	0.031 ± 0.002	
Kidneys	0.195 ± 0.007	0.146 ± 0.035	0.190 ± 0.015	0.197 ± 0.000	
Liver	0.064 ± 0.008[c]	0.116 ± 0.033[a]	0.098 ± 0.012[b]	0.139 ± 0.004[a]	P < 0.033
Lungs	0.147 ± 0.016[a]	0.123 ± 0.021[a]	0.115 ± 0.006[ab]	0.075 ± 0.004[b]	P < 0.046
Muscle	0.003 ± 0.000	0.003 ± 0.001	0.004 ± 0.000	0.005 ± 0.000	
Pituitary	0.018 ± 0.015	0.004 ± 0.000	0.003 ± 0.001	0.002 ± 0.000	
S. Intestines	0.019 ± 0.003	0.026 ± 0.001	0.014 ± 0.001	0.022 ± 0.003	
Spleen	0.037 ± 0.011	0.107 ± 0.034	0.061 ± 0.011	0.082 ± 0.001	
Testes	0.005 ± 0.001	0.005 ± 0.001	0.003 ± 0.000	0.003 ± 0.000	
Urine	0.000 ± 0.000	0.070 ± 0.069	0.000 ± 0.000	0.003 ± 0.002	

Values are the mean %kg-dose/gram ± standard error of the means (S.E.M.) for 3 rats per time period. Each animal received approximately 4 uCi of I-125 labeled corticosterone (i.v.).

(a, b, c) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

Table 20. Tissue Distribution of Iodine-125 Labeled Corticosterone in Heat-Stressed Male Rats Exposed From 5 to 60 Minutes

Tissue	Time Period in Minutes 5.00	15.00	30.00	60.00	L.S.D. at 0.05
Adrenals	0.111 ± 0.004	0.082 ± 0.011	0.032 ± 0.008	0.138 ± 0.090	
Blood	0.032 ± 0.003	0.019 ± 0.001	0.016 ± 0.001	0.014 ± 0.001	
Brain	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.002 ± 0.001	
Femur	0.009 ± 0.000	0.007 ± 0.001	0.007 ± 0.001	0.010 ± 0.002	
Heart	0.023 ± 0.003	0.017 ± 0.001	0.011 ± 0.002	0.024 ± 0.017	
Kidneys	0.141 ± 0.015[a]	0.086 ± 0.003[ab]	0.050 ± 0.007[b]	0.094 ± 0.039[ab]	P < 0.07
Liver	0.102 ± 0.007[ab]	0.083 ± 0.006[bc]	0.047 ± 0.001[c]	0.121 ± 0.022[a]	P < 0.038
Lungs	0.099 ± 0.031	0.049 ± 0.002	0.035 ± 0.003	0.135 ± 0.052	
Muscle	0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	
Pituitary	0.002 ± 0.000	0.005 ± 0.004	0.001 ± 0.000	0.003 ± 0.001	
S. Intestines	0.011 ± 0.000	0.026 ± 0.011	0.042 ± 0.016	0.059 ± 0.017	
Spleen	0.071 ± 0.016	0.080 ± 0.003	0.063 ± 0.003	0.303 ± 0.174	
Testes	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.005 ± 0.001	
Urine	0.085 ± 0.014	0.089 ± 0.052	0.237 ± 0.032	0.301 ± 0.151	

Values are the mean %kg-dose/gram ± standard error of the means (S.E.M.) for 3 rats per time period. Each animal received approximately 4 uCi of I-125 labeled corticosterone (i.v.).

(a, b, c) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

Table 21. Tissue Distribution of Iodine-125 Labeled Corticosterone at Two Minutes or Less in Control Male Rats

Tissue	Time Period in Minutes 0.25	0.50	1.00	2.00	L.S.D. at 0.05
Adrenals	0.110 ± 0.008[a]	0.062 ± 0.013[b]	0.113 ± 0.021[a]	0.120 ± 0.009[a]	P < 0.046
Blood	0.146 ± 0.007[a]	0.118 ± 0.010[b]	0.085 ± 0.006[c]	0.045 ± 0.004[d]	P < 0.027
Brain	0.004 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.002 ± 0.000	
Femur	0.011 ± 0.002	0.010 ± 0.001	0.011 ± 0.001	0.010 ± 0.001	
Heart	0.057 ± 0.001	0.123 ± 0.092	0.124 ± 0.078	0.030 ± 0.003	
Kidneys	0.173 ± 0.008	0.171 ± 0.015	0.206 ± 0.022	0.211 ± 0.016	
Liver	0.056 ± 0.006	0.085 ± 0.004	0.112 ± 0.010	0.109 ± 0.008	
Lungs	0.147 ± 0.016[a]	0.114 ± 0.018[ab]	0.103 ± 0.006[bc]	0.065 ± 0.004[c]	P < 0.042
Muscle	0.008 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	
Pituitary	0.016 ± 0.011	0.003 ± 0.000	0.002 ± 0.000	0.002 ± 0.001	
S. Intestines	0.023 ± 0.004	0.020 ± 0.004	0.022 ± 0.004	0.022 ± 0.002	
Spleen	0.089 ± 0.012	0.071 ± 0.011	0.101 ± 0.010	0.109 ± 0.006	
Testes	0.005 ± 0.000	0.004 ± 0.001	0.004 ± 0.000	0.003 ± 0.000	
Urine	0.001 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.006 ± 0.003	

Values are the mean %kg-dose/gram ± standard error of the means (S.E.M.) for 3 rats per time period. Each animal received approximately 4 uCi of I-125 labeled corticosterone (i.v.).

(a, b, c) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

Table 22. Tissue Distribution of Iodine-125 Labeled Corticosterone at Periods Between Five and 60 Minutes in Control Male Rats

Tissue	Time Period in Minutes 5.00	15.00	30.00	60.00	L.S.D. at 0.05
Adrenals	0.089 ± 0.018[a]	0.053 ± 0.009[b]	0.025 ± 0.001[b]	0.024 ± 0.003[b]	P < 0.033
Blood	0.024 ± 0.001[a]	0.018 ± 0.000[b]	0.013 ± 0.000[c]	0.012 ± 0.001[c]	P < 0.002
Brain	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	
Femur	0.008 ± 0.000	0.007 ± 0.000	0.021 ± 0.015	0.065 ± 0.056	
Heart	0.022 ± 0.002[a]	0.015 ± 0.000[b]	0.007 ± 0.000[c]	0.006 ± 0.001[c]	P < 0.004
Kidneys	0.120 ± 0.007[a]	0.075 ± 0.001[b]	0.041 ± 0.004[c]	0.038 ± 0.003[c]	P < 0.014
Liver	0.129 ± 0.006[a]	0.099 ± 0.008[b]	0.053 ± 0.002[c]	0.061 ± 0.005[c]	P < 0.019
Lungs	0.052 ± 0.003	0.053 ± 0.005	0.021 ± 0.003	0.033 ± 0.004	
Muscle	0.007 ± 0.000	0.005 ± 0.000	0.004 ± 0.000	0.004 ± 0.000	
Pituitary	0.003 ± 0.001	0.001 ± 0.000	0.002 ± 0.000	0.001 ± 0.000	
S. Intestines	0.023 ± 0.002	0.021 ± 0.005	0.022 ± 0.012	0.014 ± 0.002	
Spleen	0.106 ± 0.010	0.111 ± 0.004	0.091 ± 0.010	0.106 ± 0.003	
Testes	0.004 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.004 ± 0.000	
Urine	0.064 ± 0.028[a]	0.409 ± 0.034[a]	0.229 ± 0.068[ab]	0.230 ± 0.112[ab]	P < 0.225

Values are the mean %kg-dose/gram ± standard error of the means (S.E.M.) for 3 rats per time period. Each animal received approximately 4 uCi of I-125 labeled corticosterone (i.v.).

(a, b, c) Any two means within a row with the same superscript are not significant at the five percent level of probability using the LSD test.

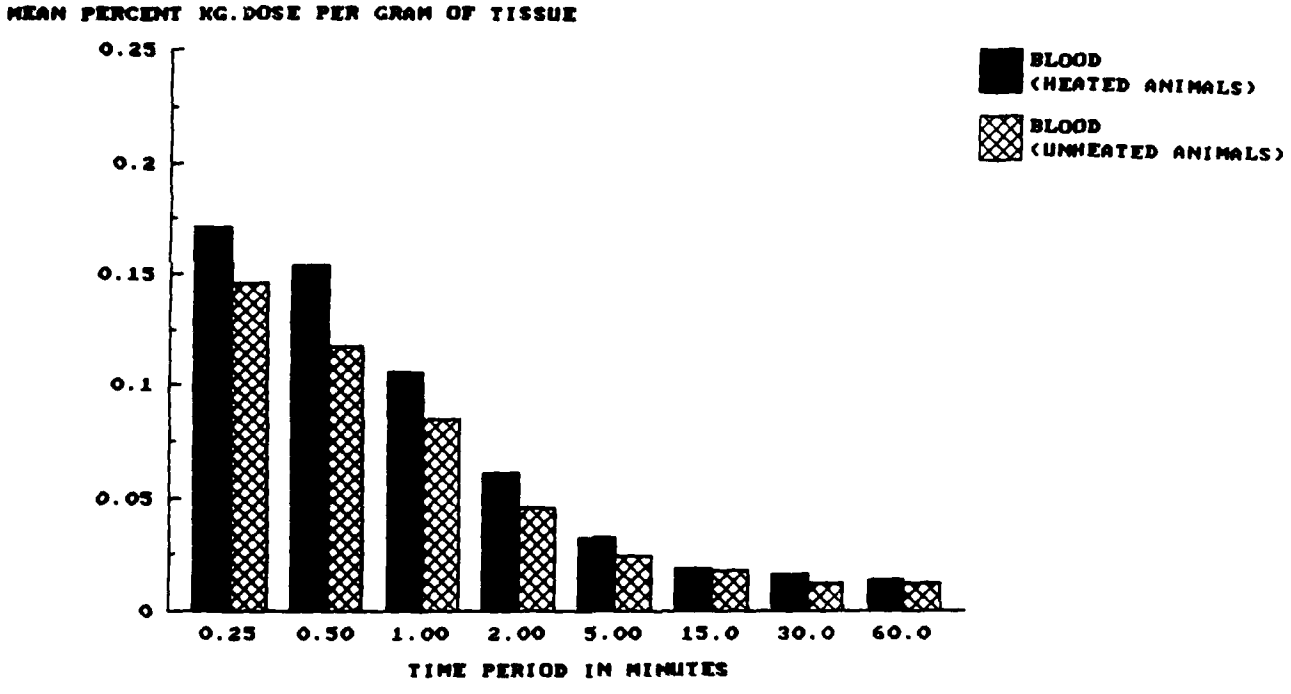


Figure 13. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

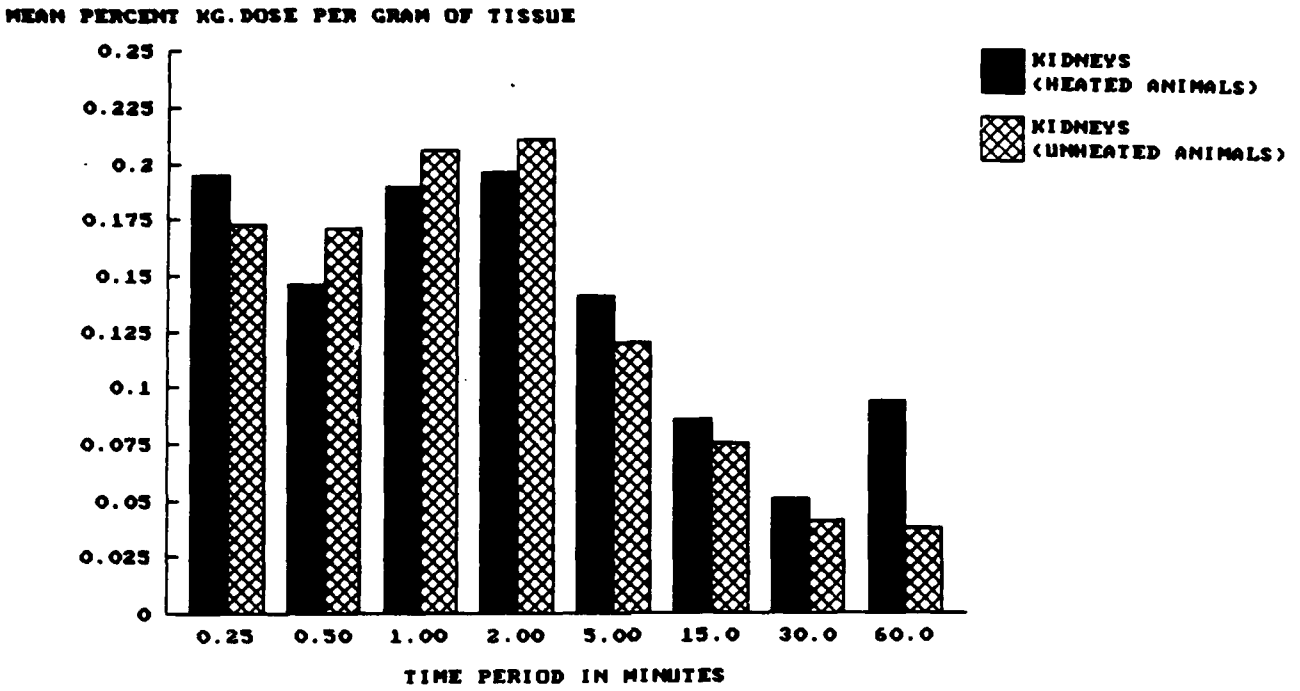


Figure 14. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Kidneys of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

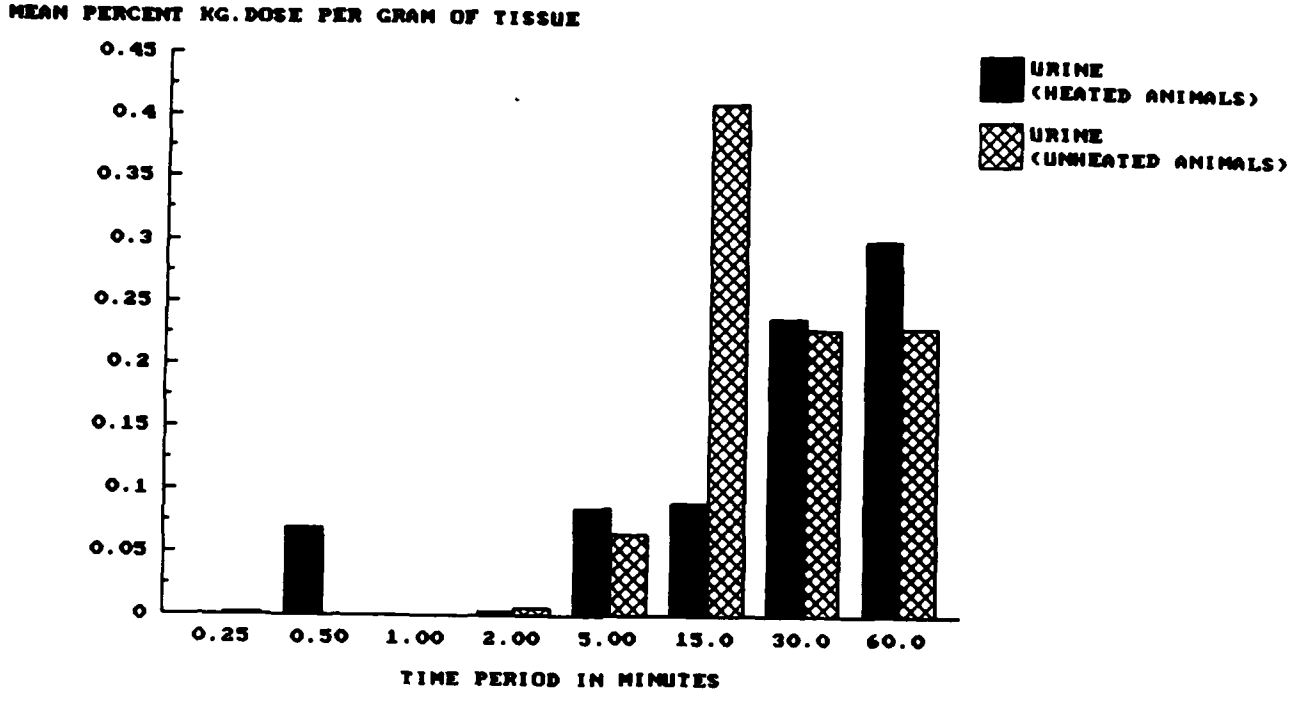


Figure 15. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Urine of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

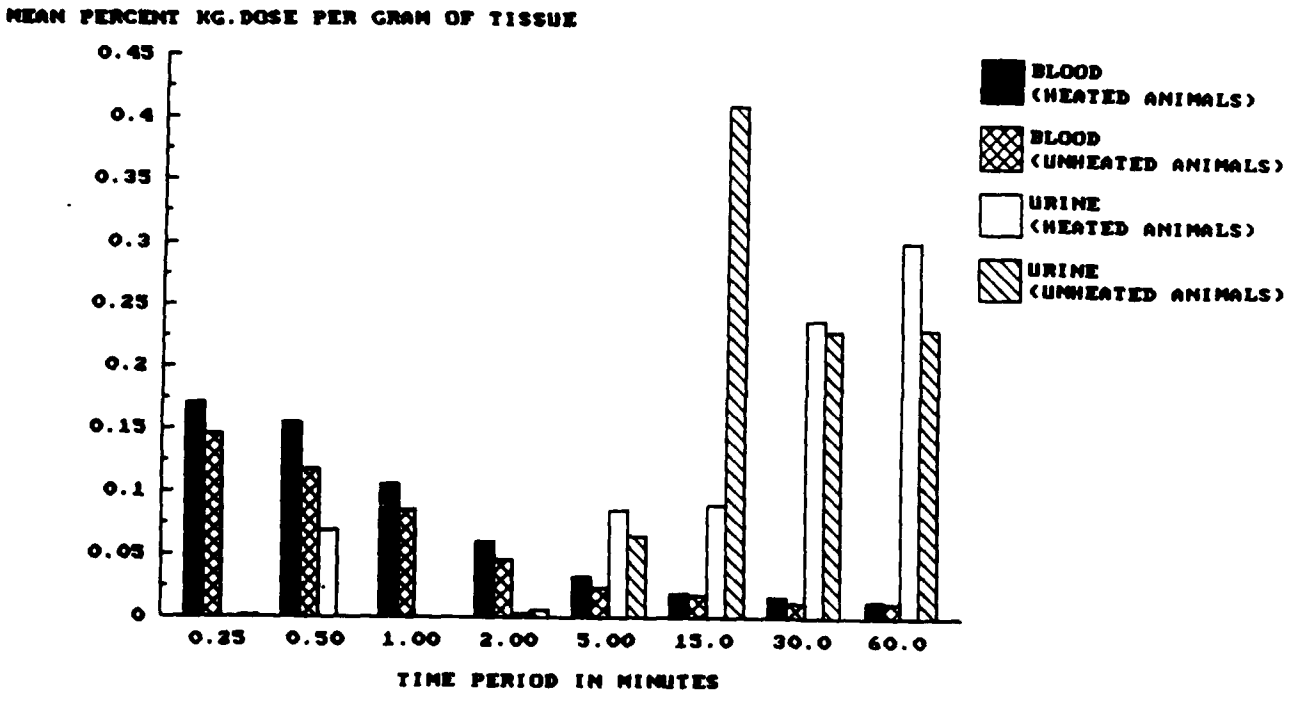


Figure 16. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood and Urine of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

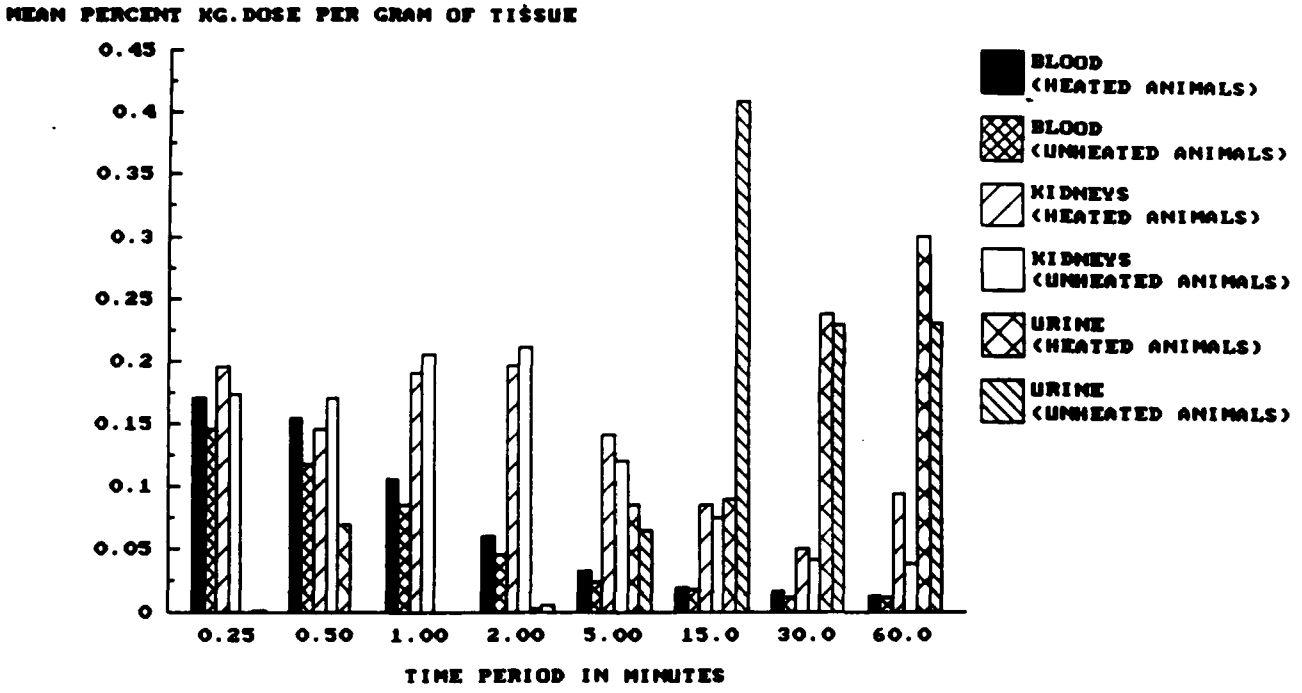


Figure 17. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Kidneys and Urine of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

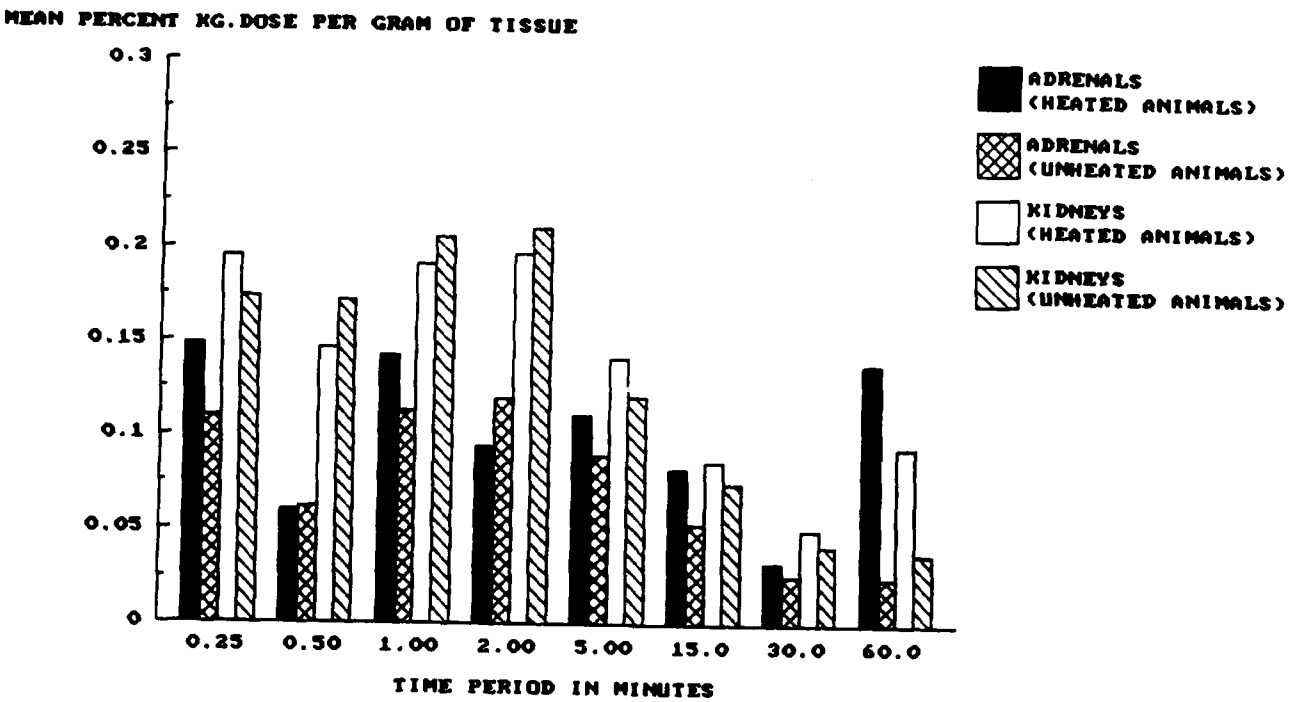


Figure 18. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Adrenals and Kidneys of, either Heat-Stressed or Unheated Control Animals at Various Time Periods.

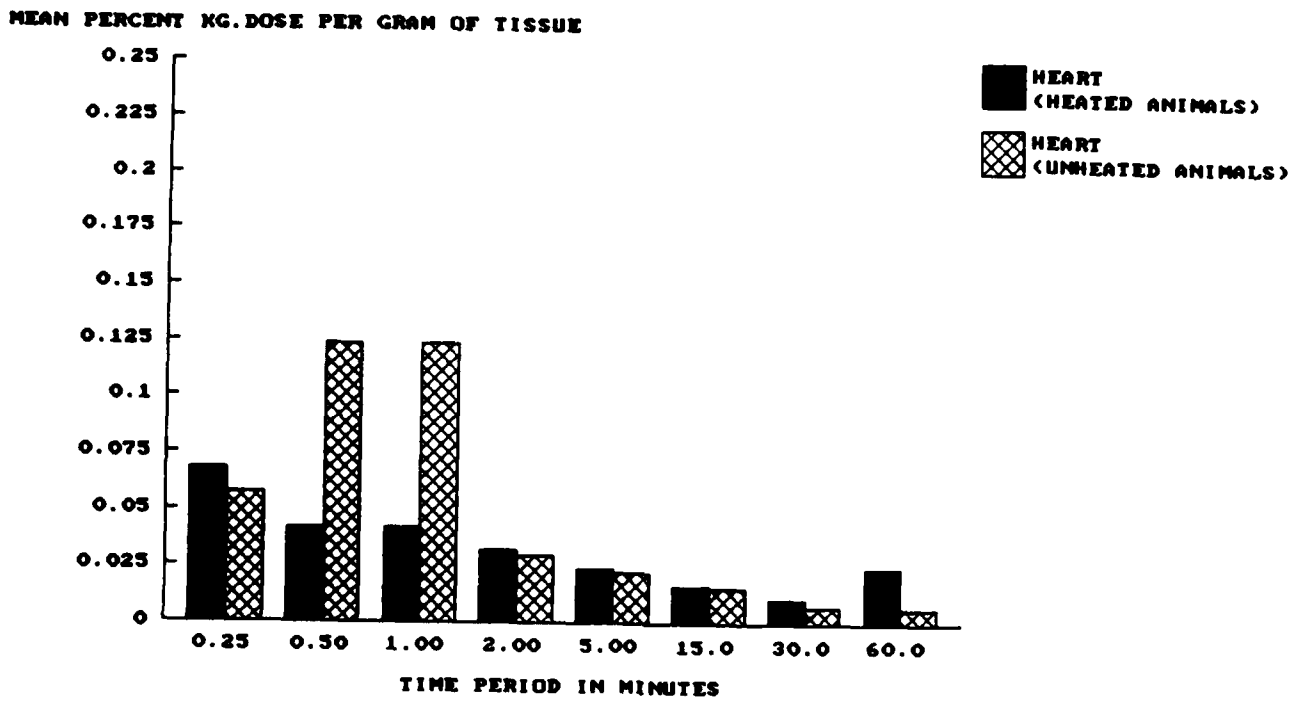


Figure 19. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Heart of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

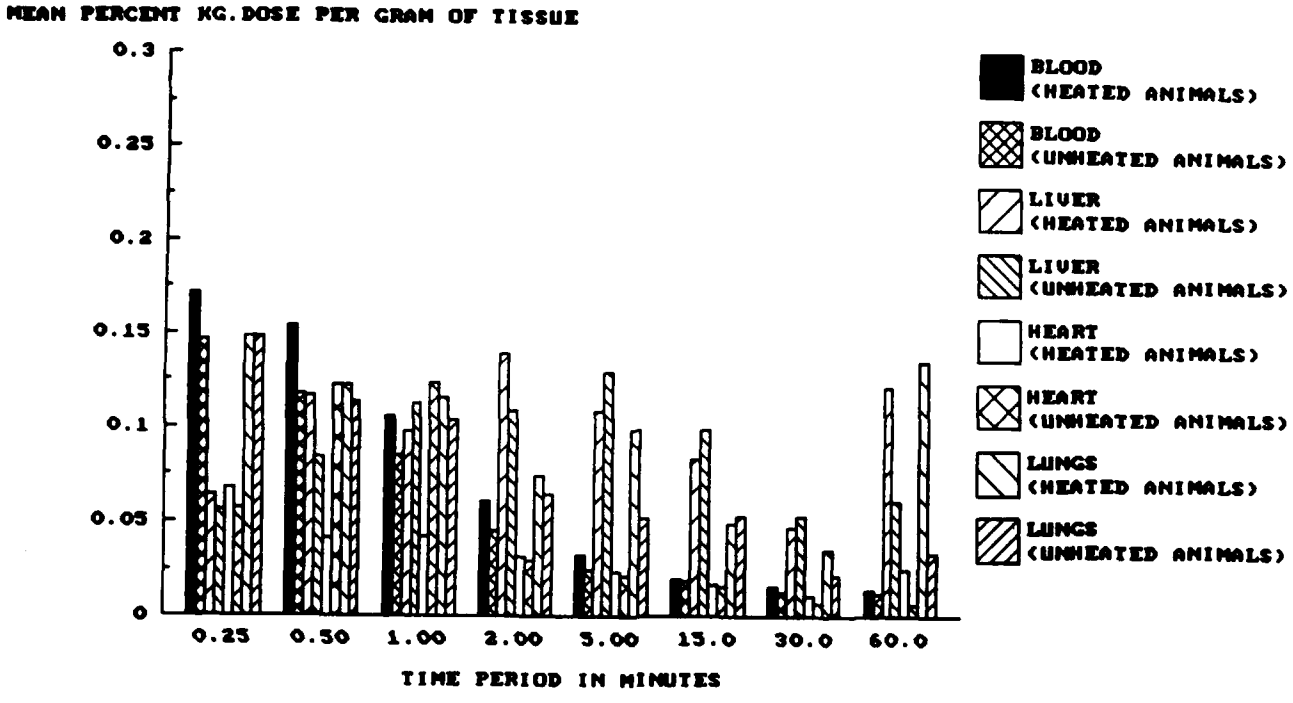


Figure 20. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Liver, Heart and Lungs of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

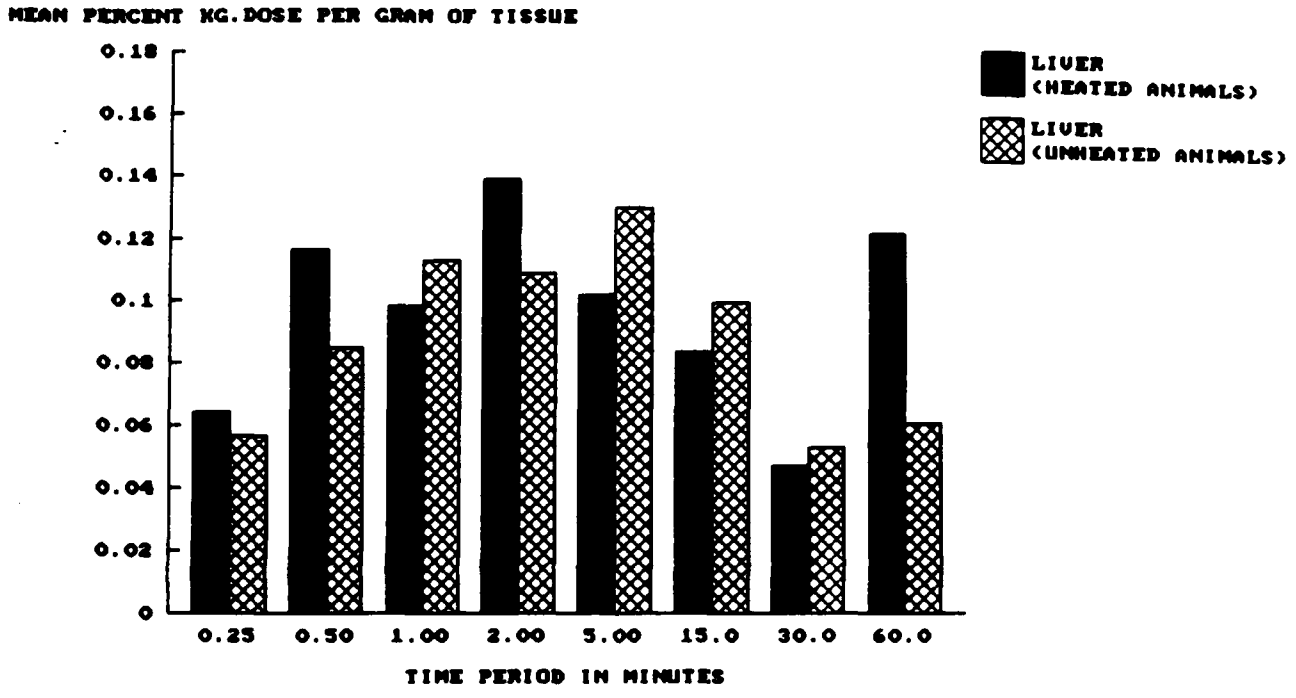


Figure 21. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Liver of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

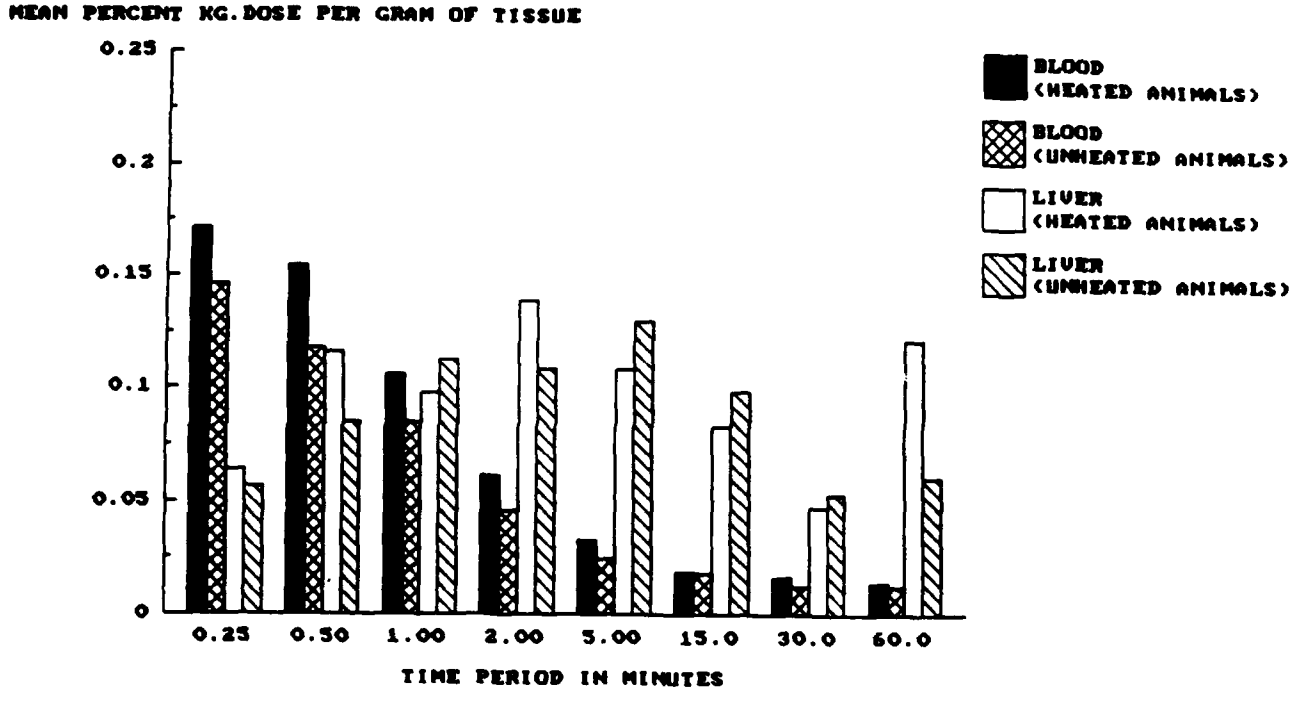


Figure 22. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood and Liver of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

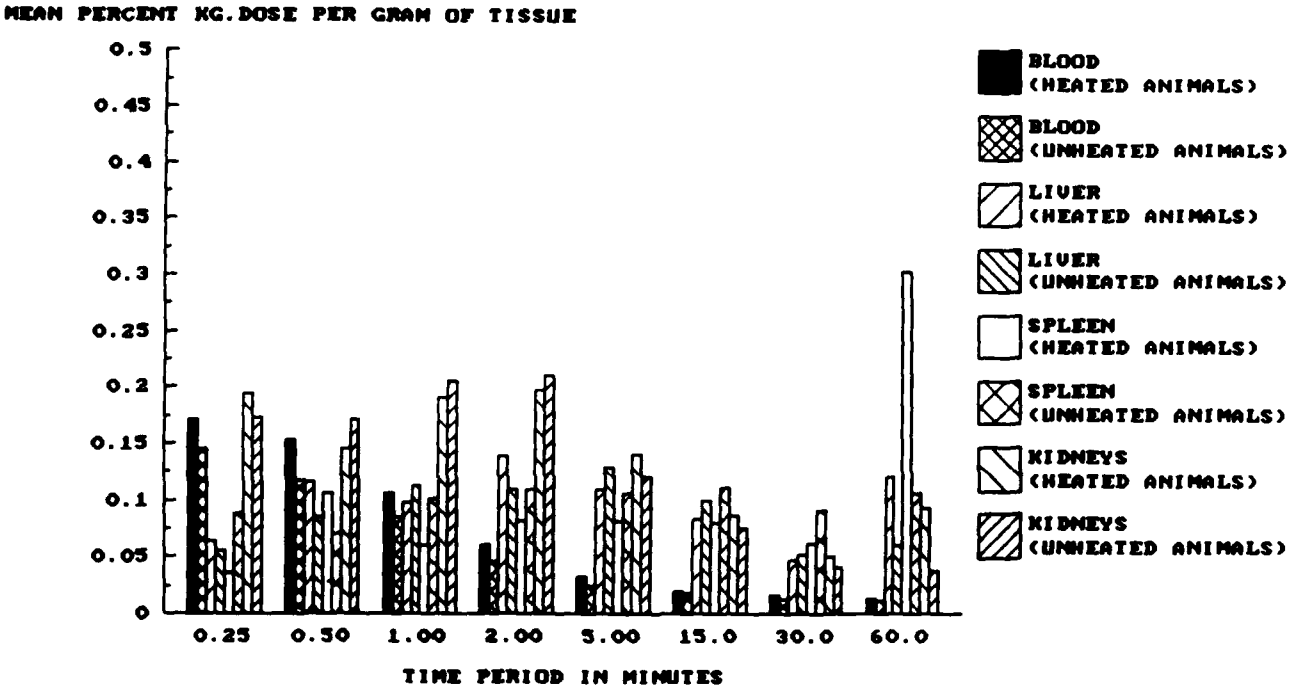


Figure 23. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Liver, Spleen and Kidneys of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

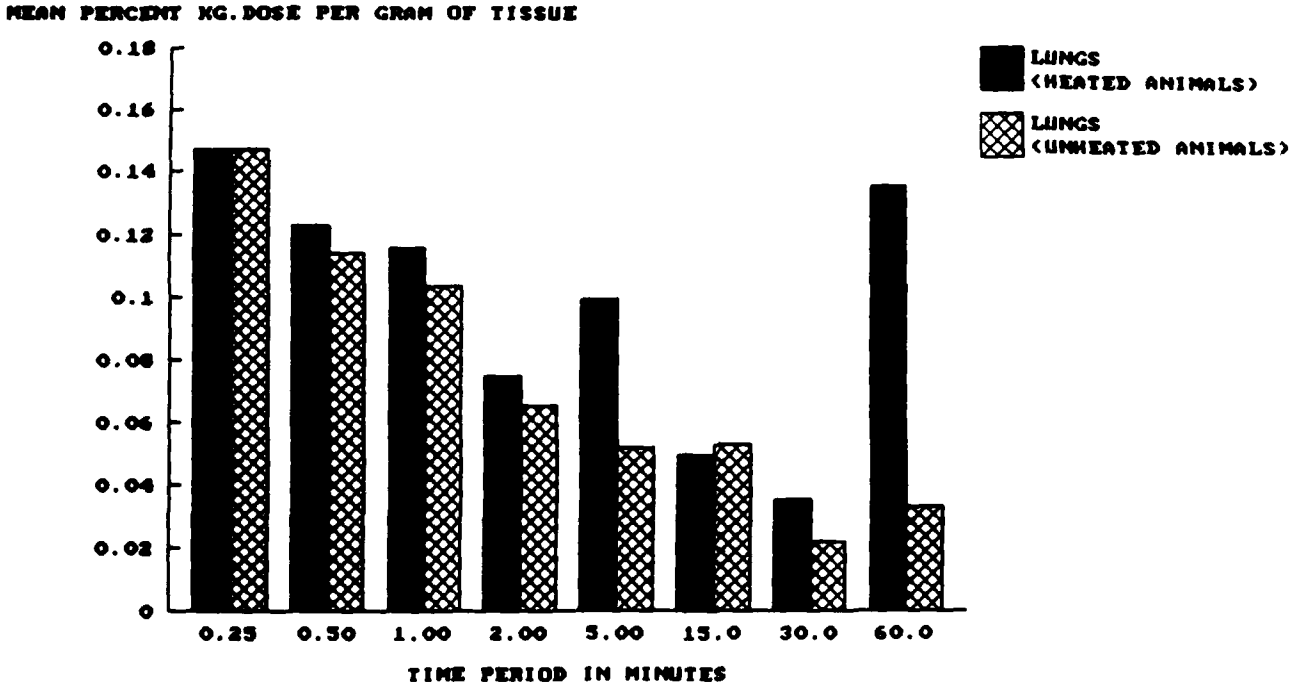


Figure 24. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Lungs of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

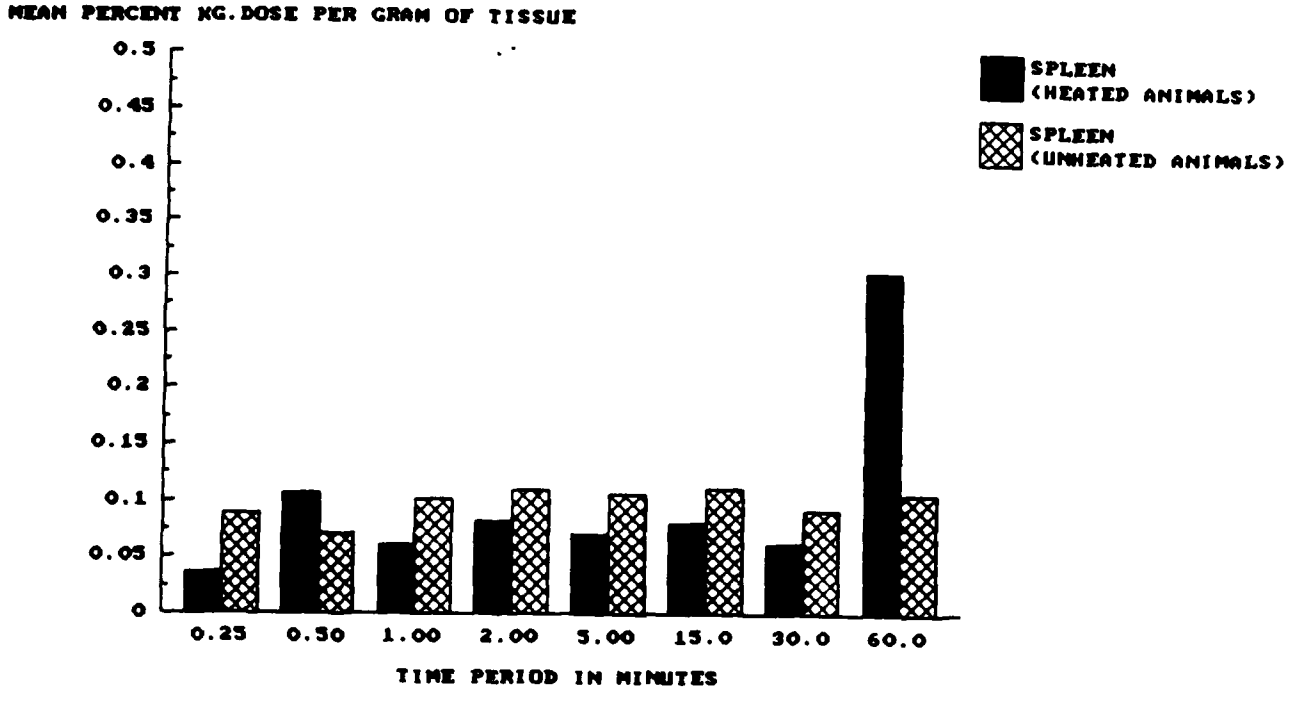


Figure 25. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Spleen of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

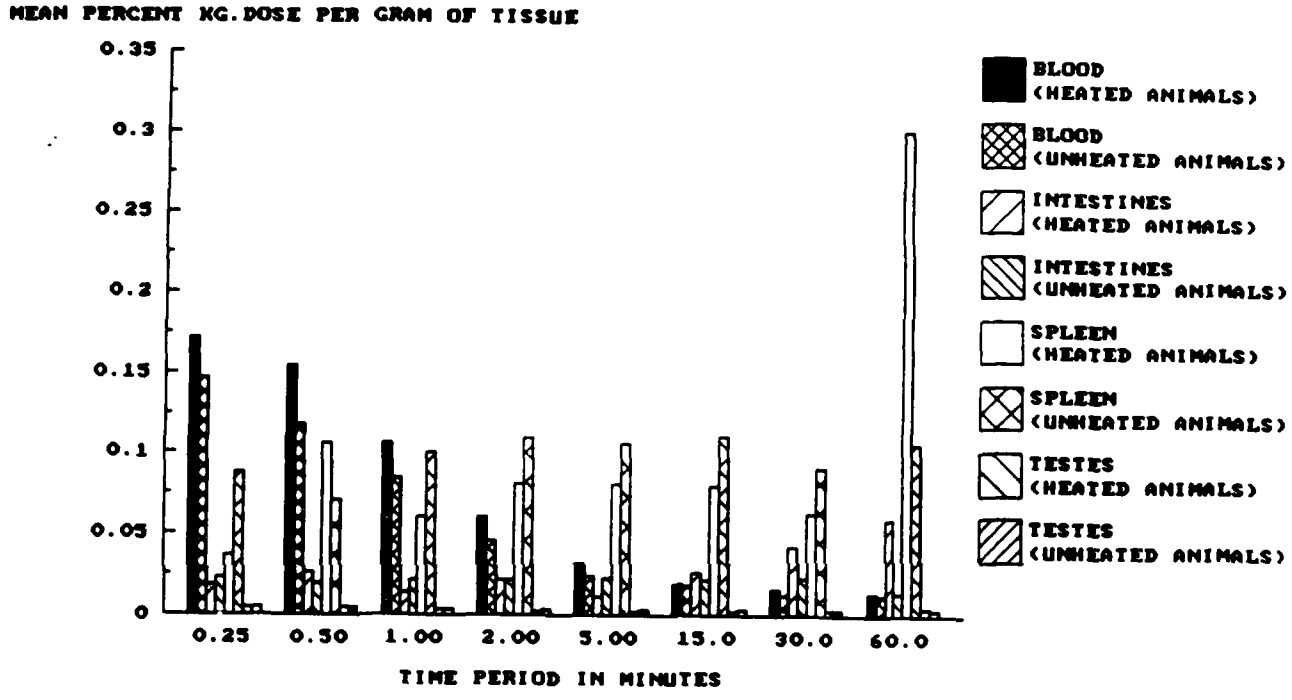


Figure 26. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Intestines, Spleen and Testes of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

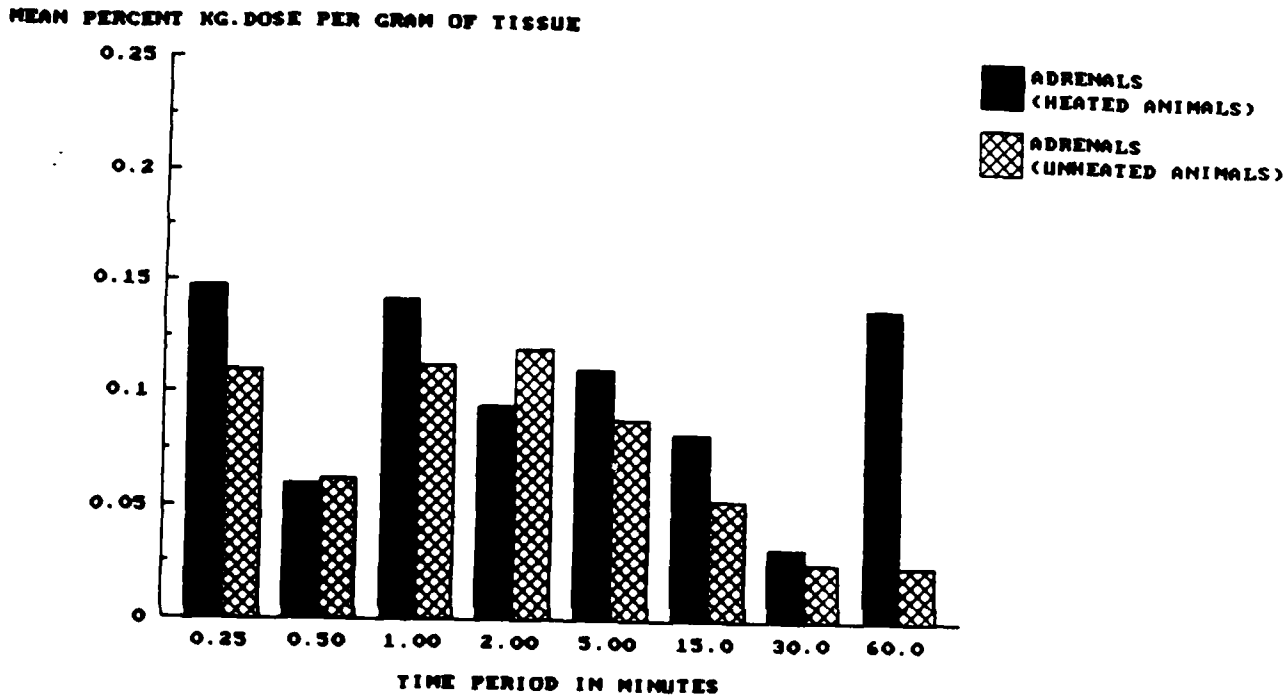


Figure 27. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Adrenals of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

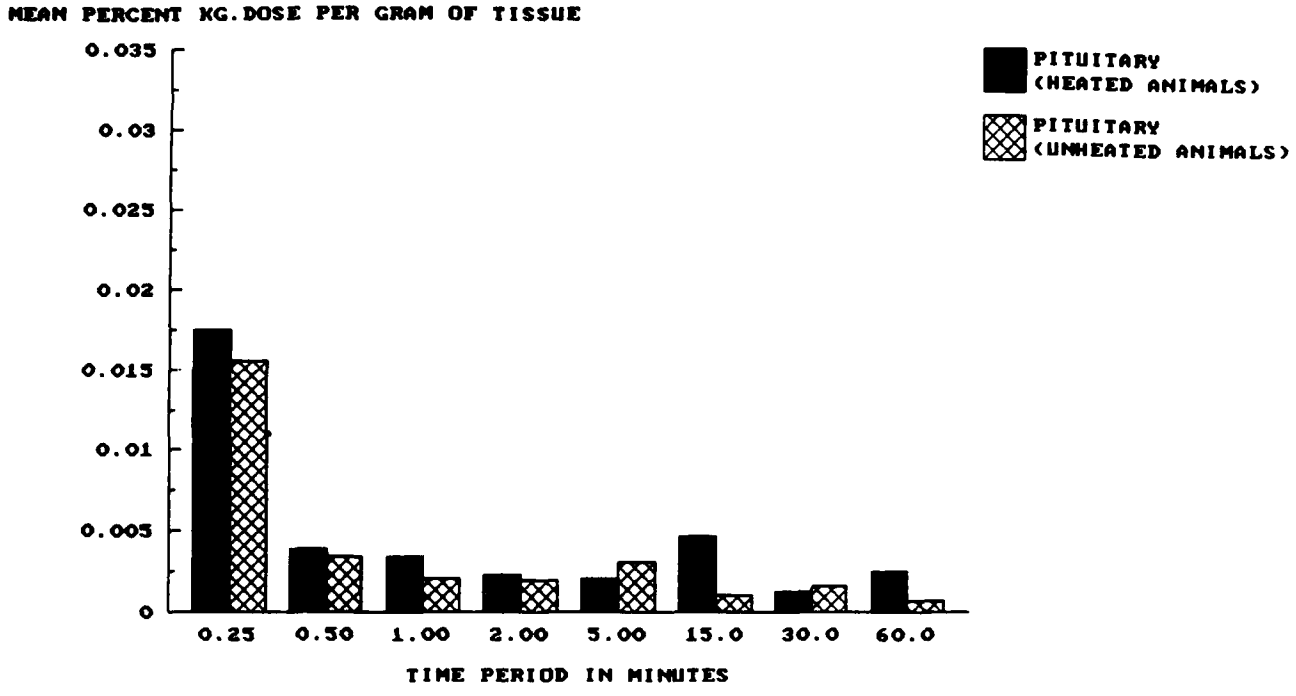


Figure 28. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Pituitary of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

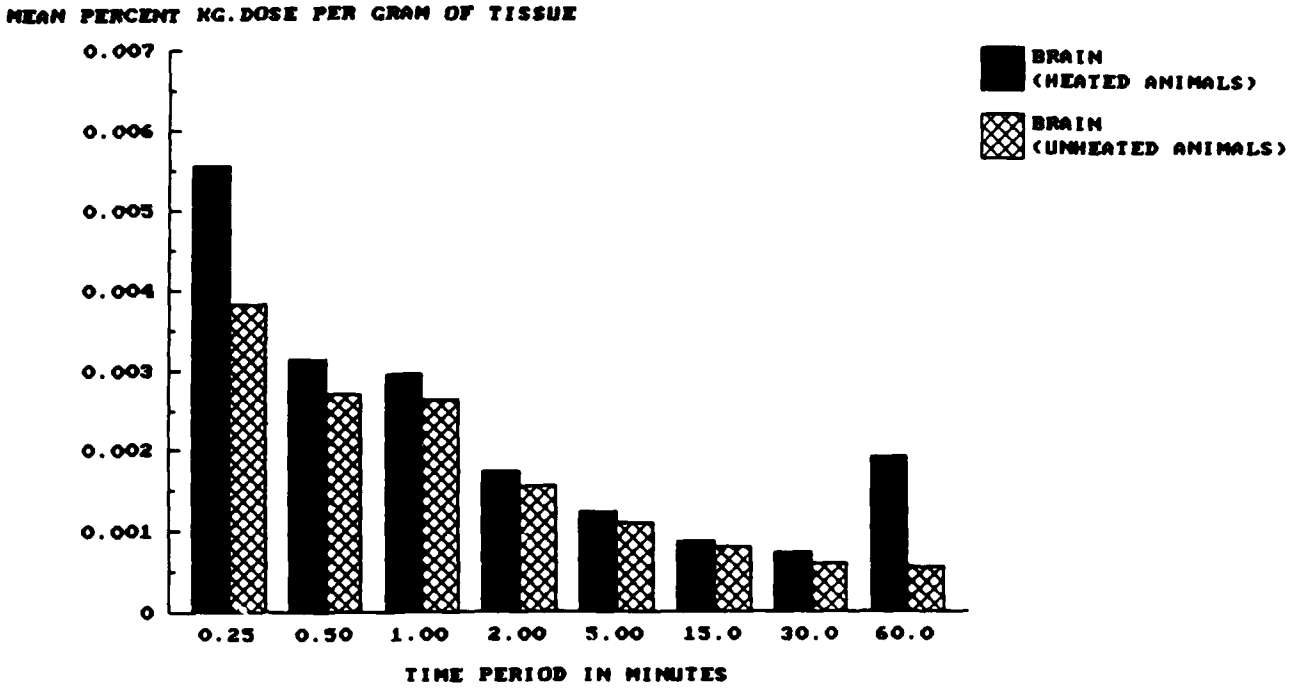


Figure 29. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Brain of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

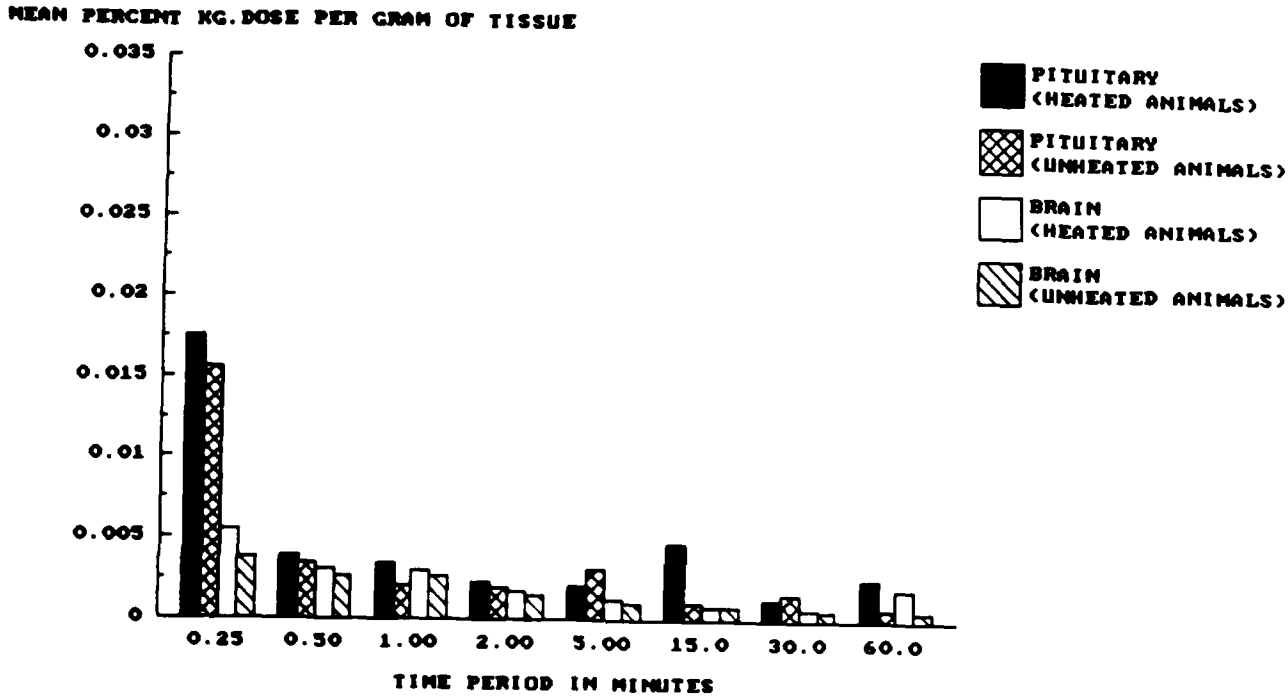


Figure 30. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Pituitary and Brain of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

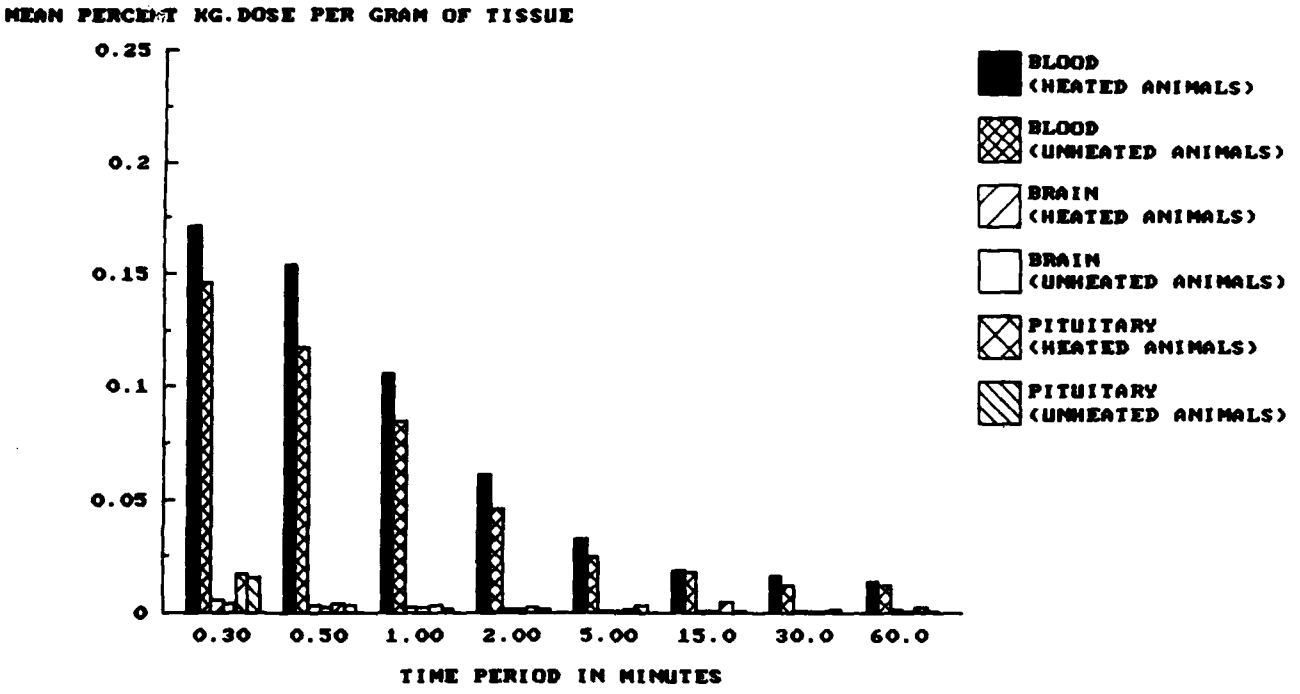


Figure 31. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Brain and Pituitary of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

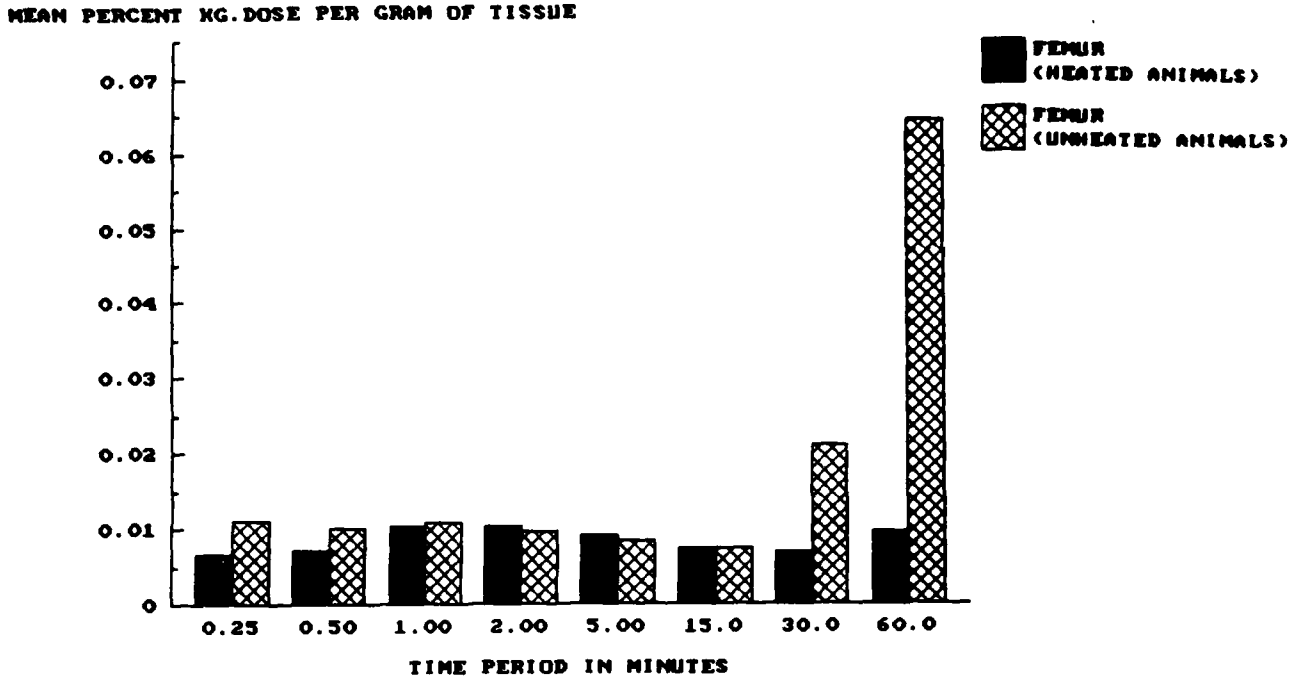


Figure 32. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Femur of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

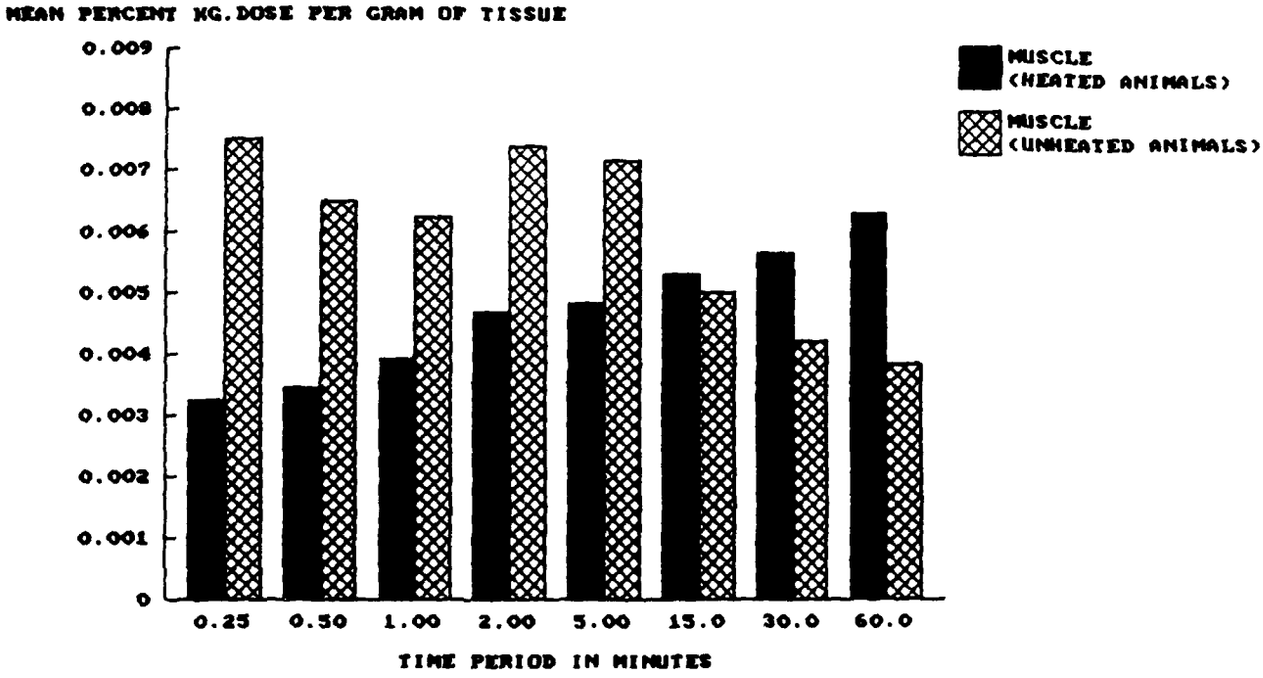


Figure 33. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Muscle of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

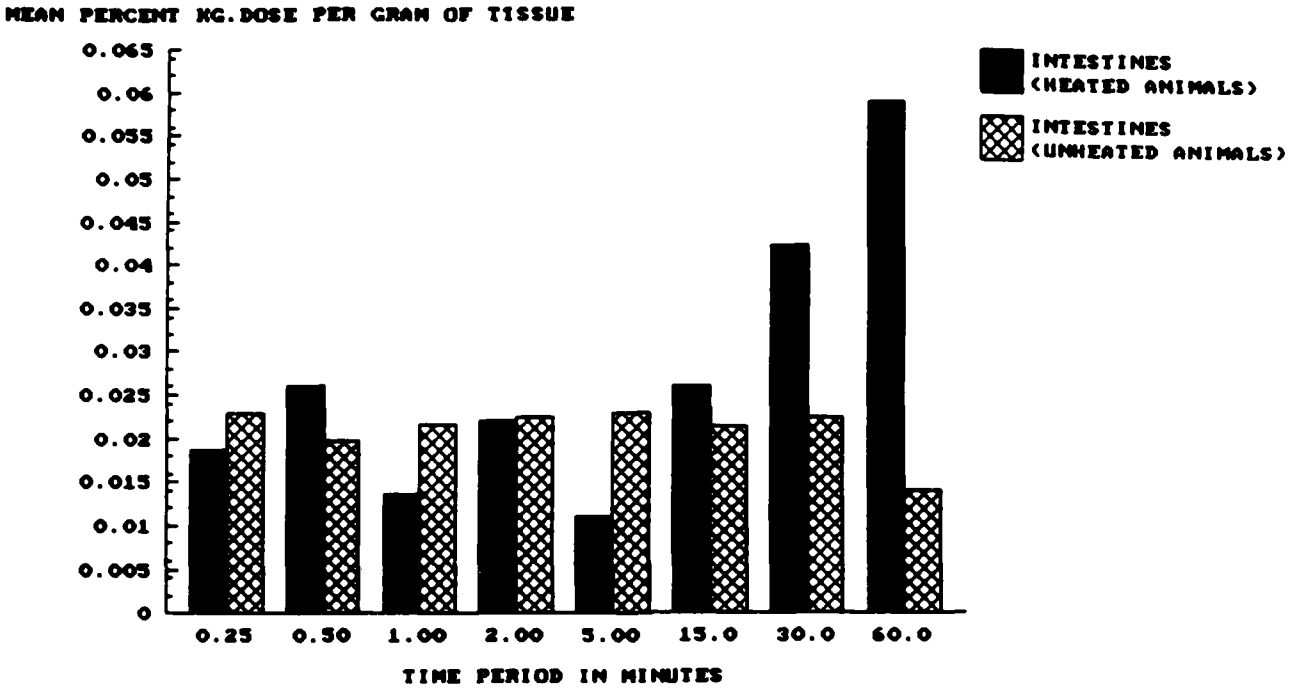


Figure 34. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Intestines of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

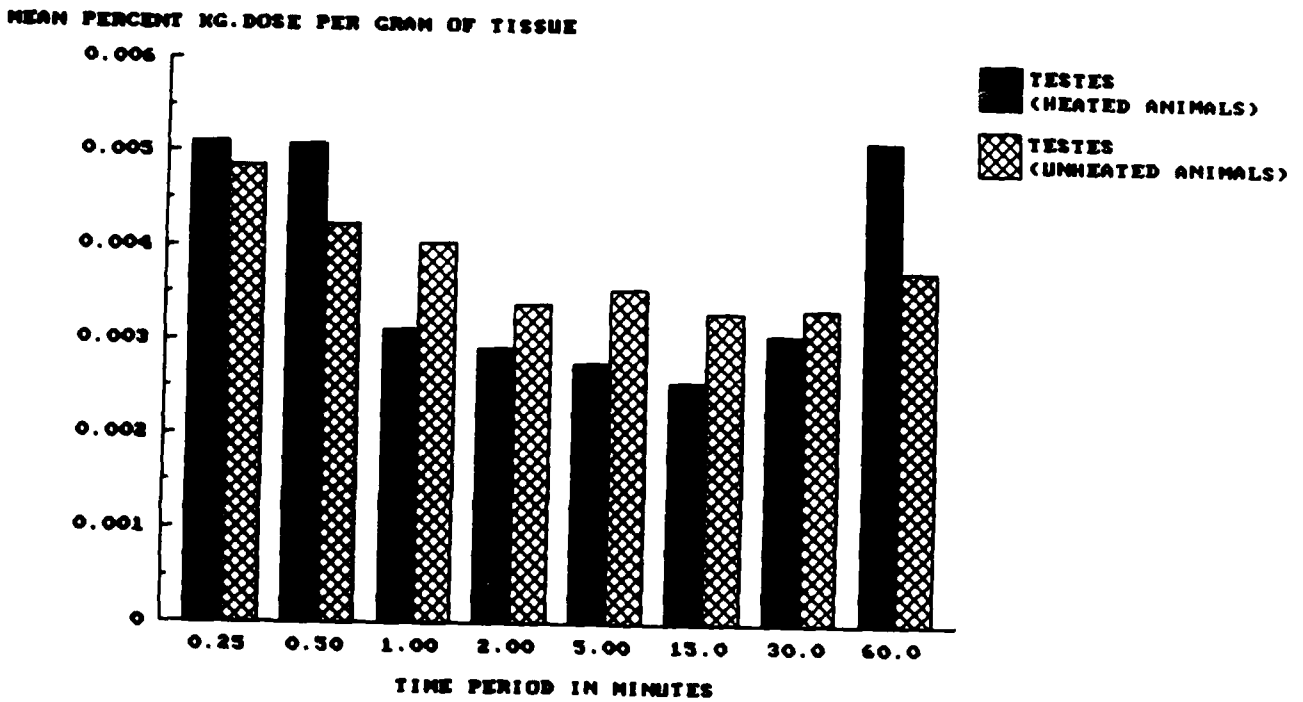


Figure 35. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Testes of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

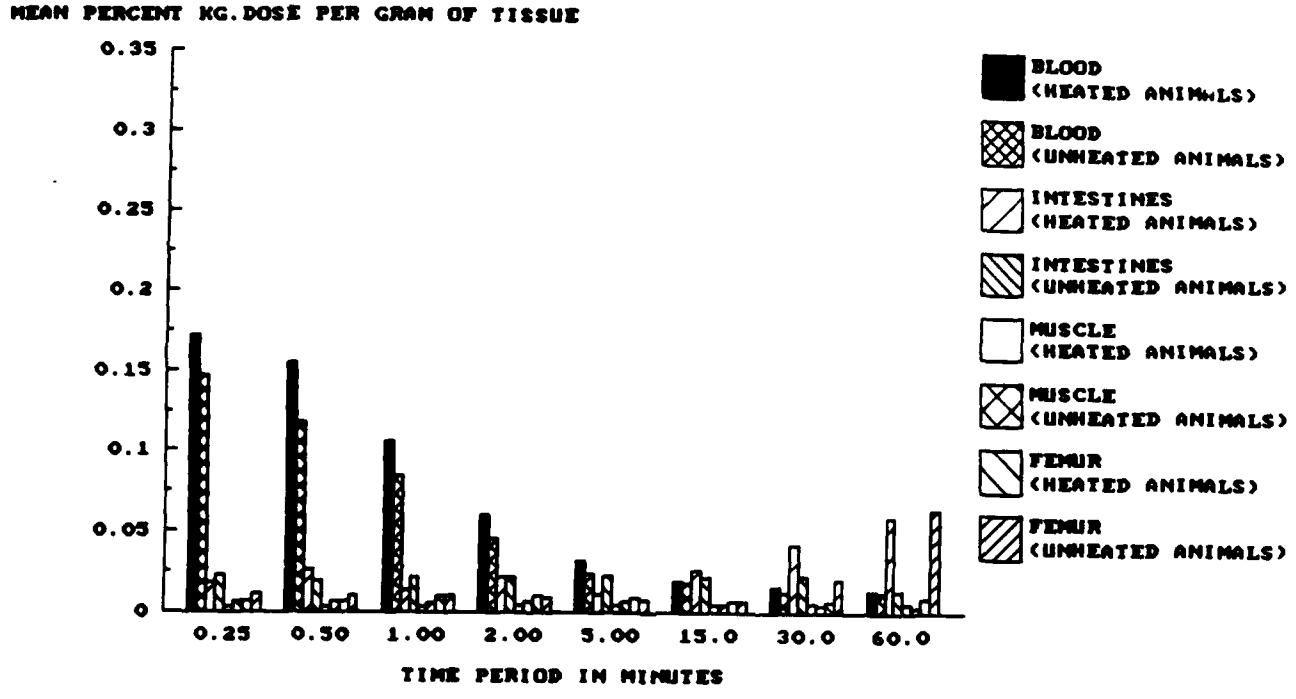


Figure 36. Tissue Comparisons of %Kg-dose/gram Values for [I-125]-Corticosterone in the Blood, Intestines, Muscle and Femur of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

E. SUBCELLULAR LIVER KINETICS OF I-125 CORTICOSTERONE IN NORMAL AND HYPERTHERMIC RATS

THE SETTING

The research team consisted of Dr. M. E. M. Tolbert, Dr. A. Weaver, Mrs. V. E. Hicks, Ms. C. Belton and Ms. A. Mauldin of the Carver Research Foundation of Tuskegee University. Experimentation in this phase of the investigations was conducted to address the possible characterization of liver subcellular distribution patterns in heat-stressed and control animals. The latter portion of this investigation was conducted at the Carver Research Foundation of Tuskegee University.

EXPERIMENTAL METHODS

Male Sprague Dawley rats (450 to 550 grams) were heated to 42.2°C to 42.6°C. The heat-stressed animals were removed from the heating chamber and monitored until their rectal temperatures dropped to or below 40.4°C. Blood samples from the heat-stressed animals were obtained by cardiac puncture when the rectal temperature dropped to or below 40.4°C. Hematocrits were also obtained from the control animals. The initial portion of the kinetic studies of I-125 labeled corticosterone were conducted at USARIEM in Natick, MA, as outlined in the previous section.

After representative tissue and organ samples had been evaluated for I-125 corticosterone content, the remaining livers from either the heat-stressed or control rats were processed and quickly frozen for further evaluation. Isolation of the liver subcellular organelles and membranes was conducted at the Carver Research Foundation of Tuskegee University. The livers were thawed, weighed and homogenized. Homogenates were fractionated into subcellular organelles. Assessments of the various subcellular fractions (i.e., nuclear, mitochondrial, soluble cytosol and microsomal), as well as that of the total liver homogenate, were analyzed for I-125 corticosterone uptake using a Beckman gamma counter. All counts are expressed as counts per minute per gram of whole liver and were adjusted for background counts.

RESULTS

The subcellular liver kinetics of I-125 labeled corticosterone were determined. As outlined in the previous section, the liver sections were obtained from either heat-stressed or control rats that were sacrificed at time intervals of 0.25, 0.50, 1, 2, 5, 15, 30 or 60 minutes after the intravenous injection of approximately four microcuries of the radiolabeled compound. Liver samples were taken from each animal and processed for the subcellular kinetics of the radiolabeled compound. The results are shown in Figures 37 - 38).

There was a rapid uptake of I-125 labeled corticosterone in the whole liver homogenate fraction with 1290 ± 312 counts per minute per gram of liver (Figure 37) at 0.25 minutes with a peak uptake at 2 minutes (2336 ± 270) and a slow decline, thereafter. When compared to the control animal liver homogenates, the heat-stressed animal liver homogenates demonstrated a more rapid uptake and longer retention of the radiolabeled compound.

There was also a rapid uptake of the radiolabeled compound in the soluble cytosol fraction of the heat-stressed at 0.25 minutes (624 ± 89 counts per minute per gram of liver (Figure 38) with a peak uptake at 2 minutes (1095 ± 270) and a decline thereafter. The nuclear fraction was variable for the livers from the heat-stressed animals. However, when compared to the control animal liver values, there was also demonstrated a rapid uptake and longer retention time of the radiolabeled compound in the nuclear fraction of the heat-stressed animal livers. The same trend was observed in the mitochondrial and soluble cytosol fractions, whereby the heat-stressed animal livers had a quicker uptake and longer retention time of the radio-labeled compound.

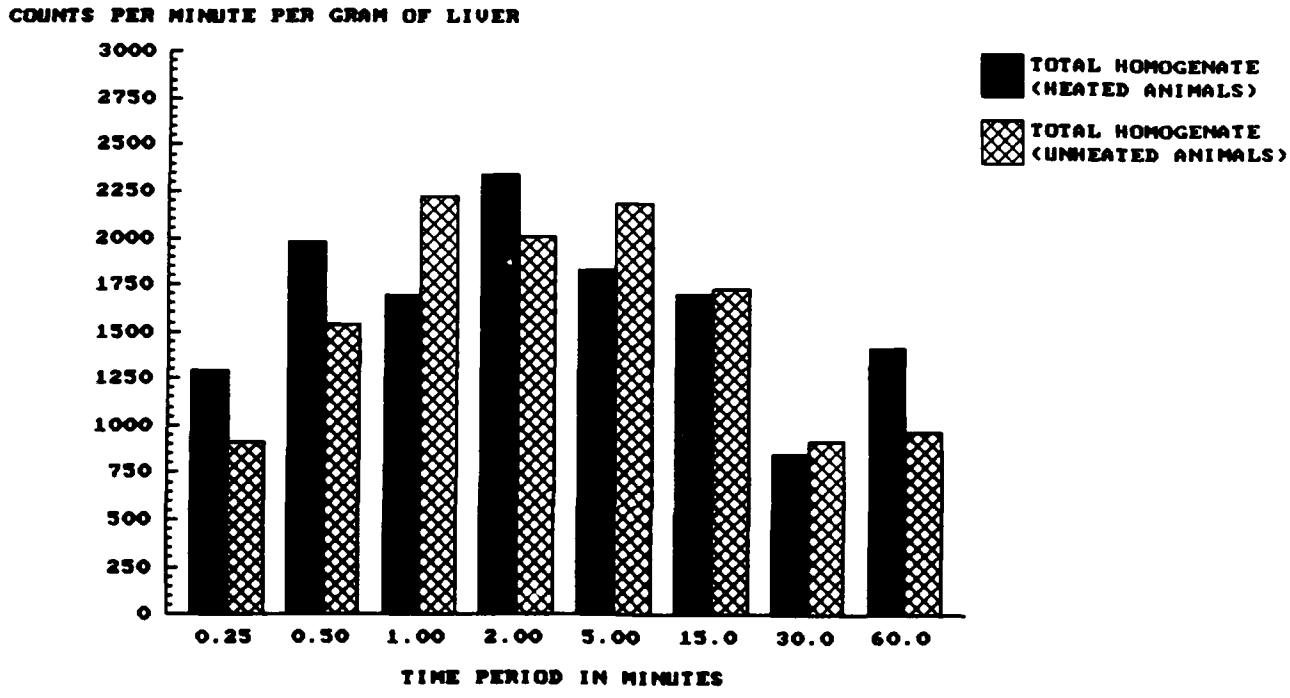


Figure 37. Liver Subcellular Localization of [I-125]-Corticosterone in the Whole Homogenate of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

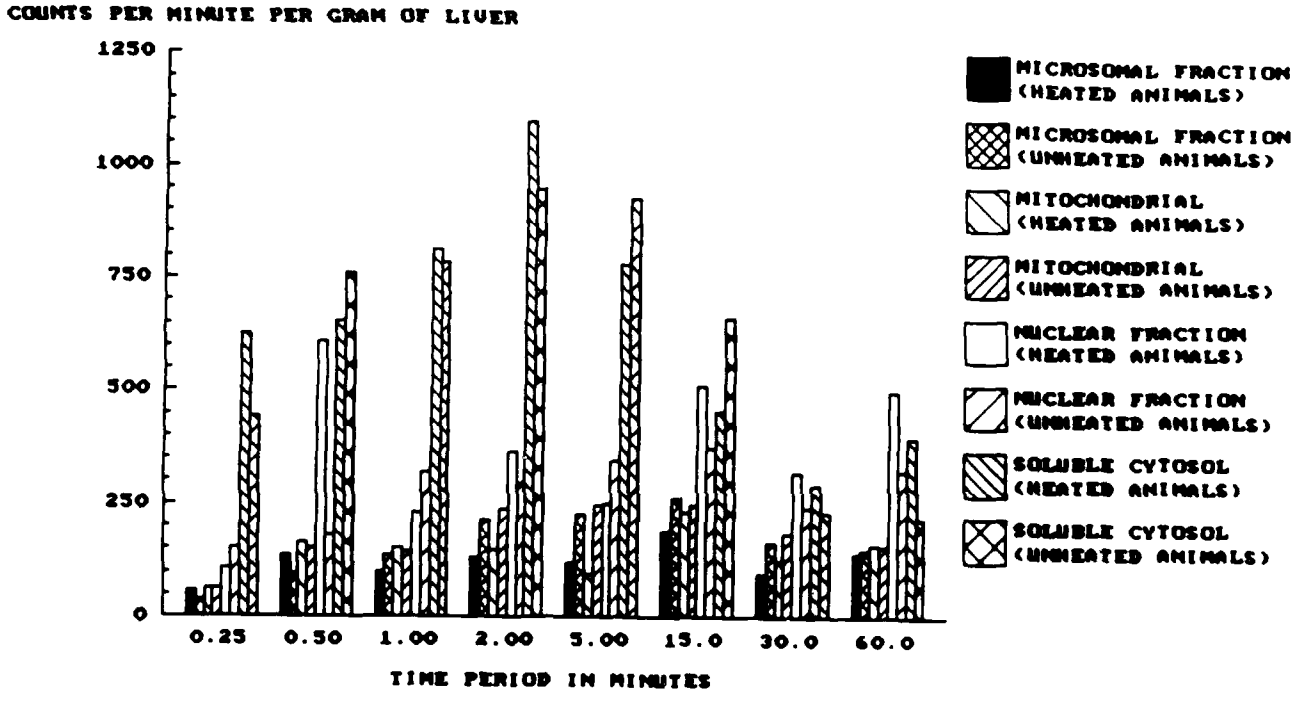


Figure 38. Liver Subcellular Localization of [I-125]-Corticosterone in the Microsomal, Mitochondrial, Nuclear and Soluble Cytosol Fractions of either Heat-Stressed or Unheated Control Animals at Various Time Periods.

DISCUSSION

Heat illness is a term which covers a wide spectrum of disorders which range from the erythematous rash of prickly heat to the extreme and often fatal medical emergency of heatstroke (8, 11, 12). The latter, the subject of the present study, is characterized by a precipitous rise in body temperature which may ultimately affect every tissue and organ. Classical descriptions of the symptoms may include elevated body temperature, central nervous system disturbances, tachycardia, hypotension, hyperventilation, respiratory alkalosis, metabolic acidosis, vomiting, diarrhea, dehydration and cardiovascular collapse (1, 8, 11, 12, 13, 15, 23, 36). Almost any combination of these symptoms, which includes hyperthermia, may occur along with the presence or absence of sweating (37).

Prevention, of course, offers the most effective approach to reducing heatstroke fatalities. However, reality confirms that the incidence of heatstroke will increase in direct proportion to the number of individuals exposed to conditions conducive to that injury. Current military strategy, which requires rapid mobilization of troops for deployment in any climate, is also subject to this axiom. For the military, this problem is further amplified by the potential requirement for the use of vehicles with no cooling capacity, the use of protective suits with no cooling capacity and the use of anti-cholinergic drugs. Predisposing factors such as heart disease (interfering with the ability of the cardiovascular system to respond) and abnormalities of the skin (interfering with sweating) represent physiological conditions which might exclude such individuals from circumstances which would result in heat overload. On the other hand, acclimatization (which increases cardiovascular and metabolic efficiency and increases plasma volume secondary to elevated levels of aldosterone relative to sodium intake), dehydration (which decreases plasma volume and increases hyperthermia) and the use of vasoactive drugs represent controllable factors requiring strict discipline. Thus, educating people at risk affords the greatest opportunity for prevention.

The actual biological mechanisms which are effectively modified to cause heat stress and heatstroke are not known. However, when one is exposed to elevated environmental temperatures, there is no set point at which one can adequately predict the onset of heat stress and heatstroke since elevated temperatures that are sufficient to produce heat stress, heatstroke or thermal related deaths are not identical. In patient studies Hart et. al. (14) reported that some patients died with rectal temperatures of 40°C while others with elevated body temperatures as high as 46.5°C and 47°C survived. Therefore, there exists the possibility of a temperature regulatory set point elevation mechanism that will cause an adjustment of thermal tolerance which is variable from one individual or animal to another. However, an overall body core temperature of 42°C or higher is considered to be incompatable with life since there exists the possibility of extensive thermal damage to internal body tissue and organ systems (35).

Although some debate continues over the best methods for rapidly lowering the body temperature of patients (1, 8, 9, 12, 19, 22, 24, 26, 31, 39), accomplishing this without inducing shivering or peripheral vasoconstriction is a main priority. Concomitant and subsequent steps relate to the obvious

AD-A182 033 THE EFFECTS OF CORTISOL CORTICOSTERONE INSULIN AND GLUCOSE PRE- AND POST-..(U) TUSKEGEE UNIV AL CARVER RESEARCH FOUNDATION M E TOLBERT ET AL. 27 DEC 86 2/2

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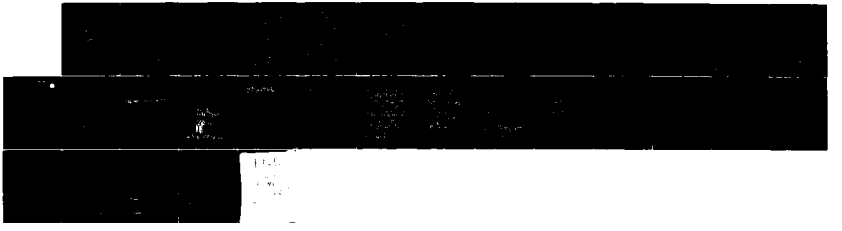

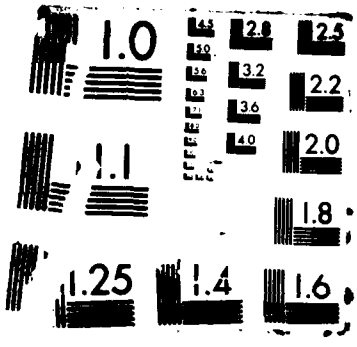
1.0
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5.0
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6.3

requirement for maintaining respiratory and heart functions (open air passage and performance of CPR, if necessary). Symptoms of pathology in various organ systems dictate what additional treatments should be included. Major considerations are:

1. Providing intravenous fluids, but avoiding fluid overload.

2. Establishing baseline clinical data such as acid-base and electrolyte balance, BUN, CPK, LDH, CBC, platelet count, liver function, arterial blood gases and urine analysis data.

3. Relieving seizures with a short-acting drug such as i.v. diazepam (some prefer chlorpromazine which can lower temperature, but may also increase the incidence of hypotension or increase the metabolic rate).

4. Reducing acute renal failure in exertional heatstroke with osmotic agents (e.g., mannitol and furosemide) when the urine is isotonic and the urine/plasma concentration ratio for urea nitrogen is less than 5:1 to prevent acute tubular necrosis.

5. Administering bicarbonate only when severe acidosis is present and while monitoring for significant hypokalemia.

6. Treating hypotension or heart failure with digitalis while considering possible metabolic acidosis and hyperkalemia (a result of inhibition of Na^+-K^+-ATPase).

7. Administering oxygen and performing bladder catheterization as symptoms dictate.

8. Using heparin if disseminated intravascular coagulopathy (DIC) is indicated.

In these situations, where the duration of pathological conditions will determine the fatality rate, prompt and comprehensive therapeutic measures are essential (8, 12).

The extent of hepatic damage, which usually accompanies heatstroke (5), provides both diagnostic and prognostic clinical indications of the severity of heat stress and heatstroke (17). This led to the use of the isolated perfused liver model by Bowers et al. (3, 4, 6) for studying heat-induced hepatic injury. Using this isolated perfused liver system, Bowers et al. (3, 4, 6) were able to address some of the questions relative to heat induced hepatic injury. Therefore, these researchers were able to use an isolated perfused liver system under conditions which allowed precise control of temperature, pH, oxygenation and perfusate flow independent of cardiovascular and hormonal influences. When this model was used to evaluate various drugs for their hepatoprotective value, the presence of insulin and cortisol in the perfusate significantly improved all measured indicators of heat-induced hepatic damage that were produced during the 90 minutes of incubation time at 42°C (3). The protective value of these compounds appears to relate to their membrane stabilizing properties.

In our efforts to study the hepatoprotective value of corticosterone, cortisol, insulin and glucose, we used isolated hepatocytes as our test system. The presence of corticosterone (at concentrations several times higher than that determined for the heat-stressed rats), low concentrations of insulin and glucose caused minor reductions in the LDH and SGOT activities of the hyperthermic hepatocytes incubated at 42°C. These effects of the drug mixtures were more pronounced up to 30 minutes of incubation. The fact that no major direct effects of the drug mixture was observed, even though high levels of corticosterone were used, may be due to the requirement of a long lag period before the liver cells would show a direct response if cultured in the presence of corticosterone. But, we did not investigate the responses of cells cultured with the drug mixtures during heat stress since we were more interested in various rapid responses (within minutes).

While conventional therapeutic regimens attack complex symptoms resulting from the failure of organ systems, the compounds in this drug mixture target cellular injury which must ultimately contribute to both organ dysfunction and fatality. A part of the rationale for our protocol is based on the concept that cell death occurs after a series of steps which progress from reversible to irreversible events (42). Intracellular repair mechanisms, humoral factors and exogenously supplied substances may intervene to prevent the irreversible stages and thus prevent cell death. Waugh (38) used cortisone in the treatment of heatstroke victims in an effort to lower body temperature and induce sweating, but he observed no beneficial effects. In the present study, we used a post-treatment drug regimen in efforts to lower the body temperature of the rats, to increase cellular viability and the overall survival rate of the rats.

An observed effect of hyperthermia in rats is an increase in endogenous corticosterone, LDH, SGOT and potassium levels (Table 15). The clinical importance of corticosterone is not sufficiently clear. The capacity to reduce ischemic damage with glucocorticoids has been well documented (10), and glucose, insulin and potassium (GIK) are used extensively in the treatment of myocardial infarctions (27, 30, 33, 41). Insulin is known to influence potassium flux, while GIK combination increases arachidonic acid formation, influences phospholipid membrane stabilization and increases prostaglandin formation. All of these effects are beneficial in reducing necrosis (19, 32). The GIK combination also increases glycolysis, decreases free fatty acids (FFA) and changes FFA composition. These changes improve electrical and mechanical stability of ischemic myocardium. In experimental anoxia, GIK also improves the production of ATP (33). According to Maroko et al. (27), the normal heart derives a substantial fraction of its energy from fatty acids and a negligible fraction from anaerobic glycolysis. However, the latter fraction increases progressively as the heart is rendered anoxic, as the perfusion medium is enriched with glucose and as insulin is added. The membrane barrier to glucose entry into the cell is overcome by high extracellular concentrations of glucose and insulin (33). GIK also restores intracellular potassium while insulin stimulates glycogen synthetase.

Evaporative heat loss from the body via the sweating mechanisms is a major non-renal route of water loss from the body. Even though the kidneys are highly sensitive to various changes in body water homeostasis, the sweating mechanisms

of the body are highly responsive to environmental and body temperature increases. Therefore, the kidneys might not be able to effectively regulate and restrict water loss during heat stress and heatstroke. The fact that the kidneys of heated rats retained more I-125 labeled corticosterone after 5 minutes of exposure indirectly supports this statement. This takes on added meaning when one considers that corticosterone is released in response to injury and that the injured organ will absorb more of the compound in efforts to off-set the damage (Figure 14). As noted in Figures 8 - 10, it seems that corticosterone, in a concentration of 49.5 ug/ml, would afford protection against heat stress and might also reduce any drug induced toxicity that could be a contributing factor to cell dysfunction and cell death. Under abnormal thermal conditions, the body is suddenly faced with the tasks of trying to both conserve body water and to maintain an adequately high evaporative body water loss in order to produce an overall cooling effect. Since the body water which is lost during the above processes is highly dependent on the functions of the various body compartments when the body is exposed to a high environmental temperature and the sweating mechanisms are activated, there will be an overall dehydration effect produced in all the tissues besides the kidneys. As noted for the kidneys (Tables 19 - 22, Figures 14, 17, 18, 23), however, it is possible that this water loss will affect the Na+ and K+ levels (Tables 9 - 16, Figures 5 - 7) as well as other ions (i.e., calcium and phosphorus) and Na^+-K^+ -ATPase levels in the circulating blood.

One must also consider that exposure to high environmental temperatures will directly influence the circulating blood and blood clotting mechanisms of the body. It is possible that heat stress and heatstroke might produce various hemorrhagic disorders (i.e., petechial hemorrhages) as well as to produce a decreased blood coagulation time and bleeding diathesis. Therefore, these blood disorders would highly influence the metabolic processes of the various organs and tissues of the body (e.g., liver, lungs, spleen and femur).

As noted (Tables 19 - 22, Figures 20 - 26, 32, 36), there is an increased uptake of I-125 labeled corticosterone in the liver, lungs, spleen and femur of the heat-stressed rats that can be critically influenced by the circulating blood. Since heat stress and heatstroke will cause the above hemorrhagic disorders, it is possible that there will be an increased number of damaged red blood cells in the circulation that are being retained by the various tissues and organs (Tables 19 - 22, Figures 13 - 36) that are either necessary to synthesize and metabolize the red blood cells or are highly dependent on the blood flow for metabolic processes.

It is interesting to note that in the I-125 labeled corticosterone studies, there seems to be a time course uptake of the radiolabeled compound in the whole brain (Tables 19 - 22, Figures 29 - 31). As a point of reference, there seems to be a rapid uptake of the radiolabeled compound at the 0.25 minute post-injection time period in both the heat-stressed and control animals with the highest uptake seen in the heat-stressed animals (Tables 19 - 22, Figures 29 - 31). However, as time passed and the heat-stressed animals were allowed to cool during the course of these investigations there was a rapid decrease in the amount of the radiolabeled compound in the brain (Tables 19 - 22, Figures 29 - 31). The decline of the radiolabeled compound in the brain might be indicative of a brain cooling effect as well as a rapid metabolism of the drug by the brain which is time dependent and might account for the lower uptake of the radiolabeled compound at the 0.50 to 60 minute post-injection time periods. If

this is true, then a concept of selective cooling of the brain or other tissues (i.e., liver and stomach) might be an important consideration in order to circumvent the deleterious effects of heat stress and heatstroke.

Our results may be consistent with the concept presented by Malamud et al. (25) that a high heat overload would cause a direct thermal damage to various target tissues (i.e., brain). The thermal damage that is produced would result in a failure of the sweating and thermoregulatory control centers, as well as to affect various internal body tissue and organ enzymatic and metabolic processes.

It is possible that exposure to high environmental temperatures will cause heat stress and heatstroke, will subsequently cause an incidence of hyperventilation and cause respiratory alkalosis due to heat exhaustion.

As noted previously, heat exhaustion will also produce metabolic acidosis (lactate acidosis) with elevated serum lactate levels. It is also possible that these increased metabolic conditions, as well as others (i.e., hypocapnia, hypotension and hypoxemia), will also produce liver impairment. A heat related impairment of liver function would account for the observations in these investigations whereby the heat-stressed animals or heat-stressed isolated liver cells demonstrated shifts in the various liver metabolic processes. These observations were further verified when we used liver subcellular fractions to investigate the liver localization patterns of I-125 labeled corticosterone in heat-stressed or control rats (Figures 37 - 38). Therefore, lactate acidosis will ensue if there is sufficient impairment of liver and lung functions since these organs will have a reduced ability to metabolize and dispose of lactate from the body. This would possibly account for the results shown in Figures 8 - 10 where there was a greater glucose production when the liver cells were incubated at 42°C in 10 mM lactate, then in 5mM glucose or no glucose, at 42°C for 30, 60 or 120 minutes in the various drug mixtures, as compared to the 37°C incubation temperature.

Injury due to myocardial ischemia and heatstroke share several common sequelae which provide further evidence tending to validate the present approach. The earliest change in either case is believed to be a loss of membrane integrity (3, 21, 30). Cardiovascular failure may determine lethality in either case. Reversal of potassium leakage also improves tissue integrity in either case. With these effects in mind and in a natural progression of our experimental approaches, we have post-treated rats with insulin, glucose and either cortisol or corticosterone after exposing them to a severe heat load to determine whether or not this post-treatment improves 24-hour viability. It appears that viability can be improved after *in vitro* post-treatment of our animal model with a mixture of corticosterone, insulin, and glucose. Our *in vivo* studies support this statement. Our experimental results show that the number of control rats (15%) which died after heat exposure was three times greater than the number which had been post-treated (5%) after heat exposure. Note that i.v. injections were used. When the therapeutic regimen was administered intraperitoneally, as was the case in our preliminary studies, 50% of the untreated rats died within 24 hours. In using either method, helpful effects were seen with extremely low concentrations of the insulin and corticosterone and even when only the drug vehicle was administered. However, i.v. administration of the therapeutic regimen is the superior route of treatment. In an effort to determine any possible interrelationships in the total body distribution patterns of the heat-stressed and control animals

(Tables 20 - 23, Figures 13 - 36), we investigated the kinetics of corticosterone uptake in normothermic (control) and hyperthermic (heat-stressed) rats. Hyperthermic rats distributed I-125 labeled corticosterone, which was given intravenously, somewhat differently then did the unheated control (normothermic) rats. Most organs and urine of the hyperthermic rats retained significantly more of the radiolabeled compound after 30 and 60 minutes of exposure than did the control rats (Tables 19 - 22, Figures 13 - 36).

These observations of the various effects of heat stress on the blood serum, as well as liver function and metabolism, are supportive of what Bowers et al. (3, 4, 6) reported. These investigators did an extensive evaluation of the liver subcellular effects of heat stress using electron microscopy to delineate any cellular alterations. We, however, used the isolated hepatic cell system, blood serum and supernatant profiles, as well as liver subcellular fractionation studies and iodine-125 labeled corticosterone kinetic modeling of the whole animals (either heat-stressed or unheated controls) as indicated in Tables 19 - 22 and Figures 13 - 38. Using these various approaches, we were also able to find some possible definitive mechanisms of the effects of heat stress on the entire animal model.

In an analysis of the radiolabeled compound uptake in the various subcellular fractions of the liver, the livers from the heat-stressed animals seem to have a greater uptake and longer retention time of the radiolabeled compound at a very early time when compared to the control animal data (Figures 37 - 38). These findings are consistant throughout the scope of these investigations and seem to demonstrate that various metabolic and enzymatic processes are activated during heat stress.

Therefore, we have based our experimental drug treatment design on the theory that there is a stepwise progression of cell degradation which will eventually lead to irreversible cell injury. The results of the various findings in this project have verified some of the proposed theories (7, 9, 28, 29, 40, 43), that have been projected, where a drug treatment design is very essential in the treatment of heat stress. As projected, there is a need for a drug treatment regimen that will bring about a membrane stabilizing effect with the subsequent reversal of the heat stress symptoms. However, these findings are not conclusive and more work needs to be done in this area.

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APPENDICES

TUSKEGEE UNIVERSITY ANIMAL CARE COMMITTEE
TUSKEGEE UNIVERSITY
TUSKEGEE, ALABAMA 36088

CERTIFICATE OF APPROVAL
FOR USE OF VERTEBRATE ANIMALS
IN RESEARCH, TEACHING AND DEMONSTRATION

Committee Number 86-02 Approved Yes

PRINCIPAL INVESTIGATOR: Dr. M. E. M. Tolbert

RESEARCH UNIT/SCHOOL: Carver Research Foundation

PROJECT TITLE: The Effects of Cortisol, Insulin and Glucose Pre- and Post-treatment on Heatstroke in Rats, and the Kinetics of Uptake and Cellular Response.

VERTEBRATE ANIMAL(S) USAGE FOR TOTAL PERIOD: Approximately 250 Rats, Sprague-Dawley Str.; 1½ yrs

---

This is to certify that the facilities are available to house the above-named species of vertebrate animals. The proper maintenance and general care will be provided in accordance with the guidelines of the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" and the Federal Animal Welfare Act. Experimental procedure(s) conducted on these animal(s) will be closely monitored. At the termination of the experiment(s), animal(s) will be humanely euthanatized, tissue(s) specimen(s) collected as needed and thereafter disposed of by incineration.

Kunwar K. Srivastava

Committee Chairman Kunwar K. Srivastava, BVSc, PhD, Dipl.

Approval Date: April 16, 1986.

APPENDIX 2

EXPERIMENTAL ANIMAL DATA FORM

LABORATORY ANIMAL SERVICES
SCHOOL OF VETERINARY MEDICINE
TUSKEGEE INSTITUTE

This EXPERIMENTAL ANIMAL DATA FORM must be completed by all investigators or instructors who plan to use live animals for research or instructional purposes. Federal guidelines mandate that Tuskegee Institute maintain and submit a detailed record of live animals used in research or teaching laboratories. Voluntary compliance with the Institute's Animal Welfare Guidelines is encouraged. The completed form should be forwarded to: The Director of Laboratory Animal Services, Department of Pathology, School of Veterinary Medicine.

.....

NAME (Investigator/Instructor) Dr. M.F.M. Tolbert 6/14/86 (DATE)

PARTMENT & SCHOOL Carver Research Foundation 727-8246 (TELEPHONE#)

TITLE OF PROJECT/COURSE THE EFFECTS OF CORTISOL, INSULIN AND GLUCOSE PRE- AND POST-TREATMENT ON HEATSTROKE IN RATS, AND THE KINETICS OF UPTAKE AND CELLULAR RESPONSE

PROJECT OR COURSE NUMBER DAMD17-86-6087 From 10/28/85 To 5/15/87 (INCLUSIVE DATES)

LOCATION OF ANIMAL HOUSING FACILITY: Carver Research Foundation Basement

INDIVIDUAL RESPONSIBLE FOR CARE AND MANAGEMENT OF ANIMALS: Mrs. Vernetta Hicks

.....

(PLEASE USE CONTINUATION PAGES AS NECESSARY)

A. EXPERIMENTAL PROCEDURES: (Please check the appropriate experimental procedure/s to be used and provide the requested information)

1. x The experimental procedure/s may involve ANIMAL PAIN AND DISTRESS which will be relieved by the use of appropriate drugs: See attachments #1 and #2.

   a. Briefly describe the types of animal experimental procedures to be used:
   Animals will be anesthetized with nembutal prior to surgery.

   b. List the drugs that will be used to relieve pain:
   Nembutal

   c. List the animal species (mice, dog, goat,etc.) and the NUMBER of each to be used in the project/course:
   Approximately 250 rats

2. x The experimental procedure/s must involve ANIMAL PAIN AND DISTRESS; drug intervention would interfere with the experimental results.

   a. Briefly describe the types of animal experimental procedures to be used:
   Some of the rats to which reference is made in #1 above will be heat stressed without being anesthetized.

   b. Provide justification for using this type of animal experimental procedure
   The use of an anesthetizing agent in the proposed experiments

with that already collected at USARIEM in Natick,MA.

c. List the animal species and number of each to be used in the project/course:

Approximately 150 rats of the 250 indicated in item #1 above.

3. ____ The experimental procedure/s will involve little or no ANIMAL PAIN OR DISTRESS (animals will be used only for specimen collection, injections, and similar procedures).

a. Briefly, describe the types of animal experimental procedures to be used:

b. List the animal species and the number of each to be used in the project/course:

B. SPECIAL PROCEDURES (CONTAINMENT): (check the appropriate item if any of the following will be used in the project/course)

1. _x_ Radioisotopes
2. ____ Carcinogens
3. ____ Organisms contagious to man
4. ____ Organisms contagious to animals

C. EUTHANASIA PROCEDURES:

1. Outline the procedure and list the drug's that will be used to euthanize the experimental animals:

Nembutal

D. PRINCIPAL INVESTIGATOR/INSTRUCTOR ASSURANCE:

I agree to abide by the "Animal Welfare Guidelines" adopted by Tuskegee Institute and permit emergency veterinary care for those animals showing evidence of pain or illness (if not the intent of the experiment).

M E M. Tolbert — SIGNATURE — 4/14/86 DATE

(DO NOT WRITE BELOW THIS LINE)

RECOMMENDATIONS, COMMENTS, ETC. Care must be taken to ensure that the rats used in experimental procedure no. 2 should not have visible signs of respiratory drains or distress. Closely monitor the temperature inside the chamber and eliminate Vent. if above the limit.

REFERENCE NUMBER: _____

Clinton C. Givens — Signature (Director of Laboratory Animal services) — 4/16/86

D-121284

Carver Research Foundation of Tuskegee University

ESTABLISHED, 1940 BY GEORGE WASHINGTON CARVER

TUSKEGEE, ALABAMA 36088

OFFICE OF THE DIRECTOR

July 11, 1986

Col. Brendan E. Joyce
Commander
U.S. Army Research Institute
For Environmental Medicine
Building 42
Natick, Massachusetts 01760-5007

Dear Col. Joyce:

The following is provided to comply with the concerns of the USARIEM Lab. (Re:"Recommend Modification and Resubmission,"provided with the minutes of the May 12 meeting of the Interim USARIEM Laboratory Animal Care and Use Committee).

a. Project staff members appreciate the affirmation of its use of laboratory animals in the proposed research.

b. Kinetic Studies

Kinetic studies will be conducted using I^{125}-Corticosterone. To avoid depletion of blood volume and the stress on rats and their loss due to surgery, we propose to replace the original protocol with the one which follows. Forty rats will be acclimatized for a minimum of 72 hours at 28°C and 30% relative humidity. Twenty will be used as unheated controls. The remaining ones will be subjected to heat as described in Attachment 1, where they are heated to a temperature of 42°C-42.6°C. After these rats have been removed from the heating chamber and their core temperatures drop to or below 40.4°C, 0.5 ml of the labeled compound in saline will be injected into the femoral vein of each. Five minutes later, a 1 ml sample of arterial blood will be collected by cardiac puncture from each of four rats after anesthetization with nembutal. Ten minutes later, another four samples will be collected from four different rats. At 15, 30, and 60 minutes, the same procedure will be performed on each of four rats at each time period given. Rats will be sacrificed after each blood collection, and organ (e.g.,liver, heart,and kidneys) will be assayed for radioactive label after dissolution in soluene. For each heated rat from which blood is collected, an unheated rat will be injected (i.v.) with 0.5 ml of the labeled compound and blood and organ samples collected at the periods indicated above. Note that drug injections except nembutal will be made intravenously.

Hematocrits will be done for each rat before and after heating.

Serum will be prepared from the blood samples. Determinations of LDH, K^+, CPK, and SGOT will be completed on each sample.

The use of this protocol eliminates the need for surgery and the heating of rats containing radioactive label. No more than a total of 3 milliliters of blood will be taken from each rat during the kinetic studies. This volume does not have to be replaced with pooled rat serum or fatty acid free albumin since the rats will be sacrificed immediately after blood and organ collections. Based on the recommendations,these modifications and procedures will be utilized.

Hepatocyte Studies

Trypan Blue is routinely used as the indicator of cell viability in the research involving hepatocytes. To date, the cells isolated by our method are not used unless 80% or more of those sampled exclude the dye. Data on the baseline values of hepatic enzymes prior to heating versus post-heating of hepatocytes will continue to be collected during each experiment. Statistical analyses will be done to determine if there are any significant differences ($p<0.05$). This information will be provided in the next progress report.

Post-Treatment Studies

In the post-treatment studies, injections of drugs will be made intravenously. If possible, sterile dextrose solution and saline will be used. Also corticosterone hemisuccinate (a soluble form of corticosterone) will be used wherever corticosterone is required without the label.

c. For scientific validity, the minimum number of animals needed per test point in the kinetic studies is three. In our protocol four are to be used per test point. The mean, standard error of the mean and p values will be calculated for the data. Data will also be subjected to Analysis of Variance and the Duncan's Multiple Range Test.

d. We appreciate the affirmation of our use of male albino rats in these studies.

The results of these studies will be compared to the pre-treatment results obtained by Dr. W. Bowers of USARIEM, who is the collaborator for this project. He used the same species and most of the methods being used in this project.

e. Procedures

The procedures used in completing this project are given in Attachments 1 to 10. Most of the procedures used involving whole animals are essentially those used by Dr. W. Bowers of USARIEM when he conducted the pre-treatment studies. A part of the rationale for the proposed protocol is based on the concept that cell death occurs after a series of steps which progress from reversible to irreversible events. Intracellular repair mechanisms, humoral factors, and exogenously supplid substances may intervene to prevent the irreversible stages and thus prevent cell death. Results from past efforts also suggest that pretreatment does not increase 24 hour viability in rats (Bowers, unpublished results, USARIEM, 1984). Our present work suggests a different approach using post-treatment and preservation of cellular integrity as the goals rather than a preheating regime and a post-heating effort to lower body temperature. Considerable evidence supports the validity of this new approach. The capacity to reduce ischemic damage with glucocorticoids has been well documented, and glucose, insulin, and potassium (GIK) are used extensively in the treatment of myocardial infarctions. GIK increases arachidonic acid formation, influences phospholipid membrane stabilization and increases prostaglandin formation. All of these effects are beneficial in reducing necrosis. GIK also increases glycolysis, decreases FFA and changes FFA composition. These changes improve electrical and mechanical stability of ischemic myocardium. In experimental anoxia, GIK also improves production of ATP. According to some investigators, the normal heart derives a substantial fraction of its energy from fatty acids and a negligible fraction from anaerobic glycolysis. However, the latter fraction increases progressively as the heart is rendered anoxic, as the perfusion medium is enriched with glucose, and as insulin is added. The membrane barrier to glucose entry into the cell is overcome by high extracellular glucose and insulin. GIK also restores intracellular potassium, and insulin stimulates glycogen synthetase.

Injury due to myocardial ischemia and that due to heatstroke share several common sequelae

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which provide further evidence tending to validate the present approach. The earliest change in either case is believed to be loss of membrane integrity. Cardiovascular failure may determine lethality in either case. Reversal of K^+ leakage improves tissue integrity in either case. With these in mind and in a natural progression of previous research, we intend to post treat rats with insulin, glucose and corticosterone after exposing them to a LD_{50} heat load to determine whether or not this post-treatment improves 24 hour viability. We intend to study the kinetics of hormone uptake in normothermic and hyperthermic rats in an effort to determine the relationship of kinetics with hyperthermia and normothermia. We also intend to study the response of isolated hepatocytes to heat, with and without hormone (labeled and unlabeled) treatment and compare data from the experiments to those of intact animals to confirm the 1984 findings of Bowers and associates. This is germane to our approach.

References for the above statements can be provided upon request. I have several publications on isolated hepatocyte; those on hepatocytes appear in the Journal of Biological Chemistry, Metabolism, Proceedings of the National Academy of Science, and the Journal of Environmental Science and Health. The results of my most recent studies of the effects of acetylsalicylic acid on adult rat hemoglobin is reported in Biochemical and Biophysical Research Communications.

f. The affirmation of the procedures to minimize pain and discomfort to the greatest extent possible without compromising the objectives is appreciated.

g. Mr. Thomas D. Martin III (Safety Office, U.S. Natick Research, Development and Engineering Center) was contacted during my initial trip to USARIEM. This is noted in my Six Month Report on this project. More recently Mr. Martin was contacted by telephone in reference to information on future use of radioactive material at USARIEM by the research team. When radioisotopes are involved., Mr. Martin will be contacted again prior to and during visits to USARIEM by project staff members.

Persons involved in this research have training and experience in the proper handling of radioisotopes and devices which produce ionizing radiation. Methods they will use will be in compliance with applicable regulations.

h. The method of euthanasia will be by nembutal injection (i.p.). This method is already in use.

i. Hopefully, the above comments will clarify the concerns of the Committee in reference to the protocol and are those which conform to the proper USARIEM Memorandia (e.g., M-70-18, AR-70-18).

Sincerely yours,

Margaret E. M. Tolbert

Margaret E. M. Tolbert, Ph.D.
Director, Contract No. DAMD17-86-C-6087

cc: Dr. L. Armstrong, COTR, USARIEM
Dr. W. Bowers, Collaborator, USARIEM
Dr. G. Silver, Chairman, USARIEM Laboratory Animal Care & Use Committee
Dr. A. Weaver, CRF
Mrs. V. E. Hicks, CRF

ATTACHMENT 1

PROCEDURE FOR THE USE OF THE HEATING CHAMBER

Calibration of Heat Chamber Computer System.

1. Put thermomix and water into water bath, and set the controls of the water bath for 37°C.

2. Connect Quartz Thermometer Probe to the Quartz Thermometer (Model 28041).

3. Insert Quartz Thermometer Probe taped to Rectal Probes into the water bath.

4. Turn on the Controller, Model 4885A.

5. Turn on the Voltmeter, Model 3456A.

6. Turn on the Scanner, Model 3495A.

7. Turn on the Printer.

8. Turn on the Box-Terminal Control-Program Switch.

9. The computer print-out with responses by project staff are as follows:
   1. ENTER ID#. Type DUBO
   2. ENTER STUDY ID. Type TOLBERT
   3. DO YOU WANT TO CALIBRATE: Type YES
   4. HOW MANY TIMES TO RUN THROUGH LOOP?: Type 1
   5. WHEN BATH REACHES 37°C, HIT THE RETURN KEY
   6. WHEN BATH REACHES 40°C, HIT THE RETURN KEY
   7. WHEN BATH REACHES 43°C, HIT THE RETURN KEY

   The printer will give calibration information:

   8. ARE YOU RUNNING ON LINE: Type YES
   9. ENTER ERROR FACTOR: Type 0
   10. ENTER ALARM LEVEL: Type 47
   11. IS ALARM LEVEL FOR A HIGH ALARM?: Type YES
   12. HOW MANY CHANNELS WITH SUPPRESSED ALARM OPTION: Type 0
   13. HOW MANY SUBJECTS: Type any number between 1 and 6
   14. ENTER SUBJECTS #1 ID:
       ENTER SUBJECTS #2 ID:
       ENTER SUBJECTS #3 ID:
       ENTER SUBJECTS #4 ID:
       ENTER SUBJECTS #5 ID:
       ENTER SUBJECTS #6 ID:
   15. ENTER INTERVAL IN SECONDS: Type 60
   16. TO EXIT THE SYSTEM: turn off the box and hit the return key.

After calibration of the heat chamber computer system, insert one of the six Rectal Probes into each of six experimental rats after placing them in a restrainer cage. These restrainer cages are the same as those described in the USARIEM "Technical Report No. T6/85." by G.J. Thomas, C.B. Matthew, W.T. Matthew, and R.W. Hubbard.

The rats, which are in the individual restrainers, can now be placed in the heating chamber. The rectal temperatures of each rat will be monitored by a computer linkage. Following the heating regimen, as noted previously, remove each rat from the chamber when their rectal temperatures rise from 42.0°C to 42.6°C. Note the animal removal time from the chamber and record the rectal temperature. Proceed with the rest of the experimental procedures as outlined under their respective headings.

ATTACHMENT 2

Serum Profile

Each rat blood sample will be collected in vacutainer tubes equipped with a 20 gauge needle, via cardiac puncture. Prior to perforation of the heart, the rats will be anesthetized with nembutal (30 mg/kg, i.p.) and the external skin puncture area made aseptic with Betadine Solution. The samples will be kept in an ice/water bath until centrifuged to separate out the serum. The blood serum will be analyzed for LDH, K+, CPK, Na+ and SGOT with the aid of an auto-analyzer. Where necessary, endogeneous insulin and corticosterone levels will be determined using radioimmunoassay kits.

M. E. M. Tolbert, A. Weaver, V. E. Hicks
Carver Research Foundation of Tuskegee University
1986

ATTACHMENT 3

CELL ISOLATION: A SURGICAL PROCEDURE

1. Each rat (male, fed) was lightly anesthetized with Nembutal (Sodium Pentobarbital, 30 mg/kg, i.p.) in order to alleviate the pain of surgery.

2. With a pair of forceps, the tongue was pulled slightly to the right side to facilitate breathing. An inverted "Y" cut was made from the sternum to pelvis, first through the fur and then through the muscle layer. The muscle and fur were clamped with hemostats and rolled out to the side to expose the internal area.

3. The stomach was located and positioned to also expose the esophagus. Two sutures were applied on two different points along the esophagus. A pair of forceps were used to support the esophagus while the sutures were put into place and tied tightly. The esophagus was cut between the ties.

4. The stomach and intestines were gently pulled to the right of the abdominal cavity. The vena cava was located; a loosely tied suture was put into place around it. The portal vein was located, elevated with the help of forceps. Two sutures were put in place around the portal vein.

5. With a catheter (fitted with a cannula) a puncture was carefully made the portal vein and the catheter pushed up the portal vein. The catheter was withdrawn from the cannula, leaving it in place in the portal vein and secured to the vein with a suture. A small opening was cut in the vena cava. Immediately, Ca+2-free Krebs Henseleit Buffer (KH) was pumped through the cannula into the liver to prevent blood clotting. The cannula was tightly tied in place with the two remaining sutures.

6. Quickly after tightening the sutures around the portal vein, the suture which was placed around the vena cava was tightened to prevent the flow of solution through the opening which was cut earlier. The thoracic cavity was opened and all organs and connective tissue removed.

7. Approximately 100 ml of KH was allowed to flow through the liver and discarded. Fifty milligrams of collagenase* was added to the temperature controlled (37°C) reservoir containing Ca+2-free KH and allowed to recycle through the liver for 20 to 30 minutes. In all cases, where KH was used, the solution was gassed continuously with a mixture of 95% oxygen/5% carbon dioxide.

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Carver Research Foundation of Tuskegee University
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*The amount of collagenase used should be varied according to its activity. The largest amount used is fifty milligrams per 100 milliliters of KH.

ATTACHMENT 4

Operation of the Perfusion Aeration Apparatus

The perfusion aeration apparatus: M-18; Medical Research Apparatus Corporation, Boston, MA.

1. With the thermostat already turned on at least 20 minutes ahead of time, fill the reservoir with 200 ml of Ca+2-free KH.

2. Connect the 95% oxygen/5% carbon dioxide gas mixture to the reservoir. Turn the pump switch on and place the inlet catheter below the surface of the KH.

3. After the liver has been cannulated, as noted previously, allow the first 100 ml of perfusate to collect in the chest cavity. Aspirate this perfusate into a waste reservoir. If the liver perfuses well, there will be a substantial blanching effect of the liver. If there seems to be an inadequate liver blanching pull back on the cannula carefully.

4. Weigh out 50 mg of collagenase and dissolve it in a small amount of Ca+2-free KH and add that solution to the reservoir. The total volume of KH in the reservoir should be 100 ml. Allow perfusion through the liver to continue for 20 to 30 minutes.

5. After the 20 to 30 minutes of perfusion, stop the flow of Ca+2-free KH through the liver and remove the recirculation tube from the chest cavity. Gently remove the liver from the rat's body.

6. Place the liver in a petri dish which is partially filled with Ca+2-free KH. Gently scrape the liver with a plastic spatula to free the cells which have been subjected to collagenase digestion.

7. Filter the cell suspension through two layers of cheese cloth.

8. Wash the cells at least three times in regular KH by gently centrifuging (50 x g) the cell suspension for 2 minutes in a table top centrifuge. In the final wash, resuspend the cells in regular KH.

9. After resuspending the cells to a concentration of 50 mg of wet cells/ml of of KH, the cells are ready for immediate use if at least 80% of an aliquot of the cell suspension excludes trypan blue, a dye used to assess cell viability.

M. E. M. Tolbert, A. Weaver, V. E. Hicks
Carver Research Foundation of Tuskegee University
1986

ATTACHMENT 5

PREPARATION OF CELLS FOR USE

1. After removing the liver from the rat, transfer the perfused liver to a plastic petri dish.
2. With a plastic spatula, gently scrape the lobes of the liver to disperse the cells.
3. Filter the cell suspension through cheese cloth. (Use only about 10 to 20 ml of Krebs-Henseleit (KH) to rinse the cells.)
4. Distribute the suspension equally into plastic centrifuge tubes.
5. Centrifuge the samples 2 minutes at 50 x g with a clinical centrifuge.
6. Aspirate off the supernatant in each tube.
7. Wash cells 3 times with regular KH.
8. After the third washing, resuspend cells in regular KH and determine the wet weight of the cells.
9. Determine the amount of regular KH solution needed to make the cell suspension 50 mg of wet cells per ml of solution.
10. Test with Trypan blue for cell viability.
11. The cell suspension is now ready for immediate use if more than 80% exclude the Trypan blue.

Cell Wet Weight Determination

To determine the wet weight of the cells in suspension, weigh an empty test tube. Pipet 1 ml of cell suspension into the tube; centrifuge the suspension 5 minutes at full speed in a clinical centrifuge. Pour off the supernatant. Wipe the walls of the tube dry with a Kimwipe without touching the cell pellet. Weigh the pellet in the tube. Subtract the weight of the empty tube from the weight of the tube plus the pellet to determine the wet weight of the cells. Calculate the volume required to make the cell suspension 50 mg/ml of wet cells.

M. E. M. Tolbert, A. Weaver, and V. E. Hicks
Carver Research Foundation of Tuskegee University
1986

ATTACHMENT 6

Procedure For Handling Cells For Heat Stress Studies

1. Pipette the desired concentrations of each drug or drug mixtures into plastic test tubes (100 X 17 mm).

2. Pipette 1 ml of cell suspension (50 mg/ml) into each tube.

3. Gas each sample with a mixture of 95% oxygen/5% carbon dioxide. Cap each sample tube tightly.

4. Place the first two samples (the zero controls) of each set (2 from the 37°C set and 2 from the 42°C set) in an ice/water bath. Place the remaining tubes in separate water baths (one set at 37°C and the other set at 42°C). Incubate samples in a water bath shaker set at the above temperatures for the specified time.

5. While waiting for the completion of the 30 minutes of incubation, centrifuge the 4 tubes placed in the ice/water bath using a table top clinical centrifuge (Model IEC) for 10 minutes at top speed.

6. After the 30 minutes of sample incubations are over, remove the tubes from the water baths and place them into an ice/water bath. Centrifuge the samples using a Beckman Model T-6 Centrifuge for 10 minutes at 2500 rpm.

7. Decant the supernatant into corresponding tubes. Permit the tubes with the pellets to drain.

8. Pipette 1 ml aliquots from the supernatants into another set of corresponding tubes. To each, add 1 ml of water and analyze for the desired parameters.

9. The pellets can be analyzed for glycogen or frozen for future glycogen determinations using the anthrone method.

M. E. M. Tolbert, A. Weaver, V. E. Hicks
Carver Research Foundation of Tuskegee University
1986

ATTACHMENT 7
(AMENDMENT)

PROCEDURE FOR UPTAKE STUDIES USING HEAT STRESSED AND UNSTRESSED CELLS
(OPTIONAL)

1. Cell suspensions from either the 37°C or 42°C temperature treatment will be used in this investigation.
2. The cell suspensions from either the 37°C or 42°C treatment will be treated as follows.
3. Add the radiolabeled compound (I-125-corticosterone) to each test tube.
4. Incubate at either 37°C or 42°C.
5. Centrifuge the tubes at 20,000 x g for 1 hour in an ultracentrifuge.
6. Separate the supernatant from the cell pellets.
7. Assay the cell pellets and supernatants for the radioactive label.
8. A further fractionation of the cell pellets will be done by resuspending the cell pellets and centrifuging the suspensions at 150,000 x g for two hours with the subsequent assay of the fractionated components.

M. E. M. Tolbert, A. Weaver, and V. E. Hicks
Carver Research Foundation of Tuskegee University
1986

ATTACHMENT 8

GLUCOSE ASSAY

1. The Glucose Stock Solution is 0.01 gram glucose/ml.
2. Dilute 0.5 ml of glucose stock solution to 10 ml with water.
3. Preparation of the various Standard Solutions from the 500 ug/ml (E) standard solution.

Tube #	ml of Solution E	Water(ml)	Final Conc'n(µg/ml)
A1,A2	0.0	1.0	0
B1,B2	1.0	19.0	25
C1,C2	1.0	9.0	50
D1,D2	1.0	4.0	100
E1,E2	1.0	0.0	500

4. Pipet aliquots of each sample into the corresponding tubes. Add sufficient water to make 200 µl.
5. Pipet 200 µl of each standard, #A1 through E2 into properly labeled tubes.
6. To each standard and sample, add 0.8 ml of glucose oxidase reagent.
7. Allow samples and standards to incubate at 37^{0}C for 30 miuntes or to incubate at room temperature for 60 minutes.
8. Remove the samples from the water bath and add 2.0 ml 7.5 N Sulfuric Acid.
9. Record the absorbance of all samples and standards at 540 nm (zero the spectrophotometer with the "A" samples).
10. Determine the concentration of each sample using the recorded values for the standards.

Glucose Oxidase Reagent

Add 250 mg of crude glucose oxidase and 5 mg horse radish peroxidase (Rz = 0.3 or higher) to 50 ml of glycerol buffer. Mix well; add an additional 50 ml of the buffer. Then add 12.5 mg of O-dianisidine-di HCL. Shake to mix. The solution can be stored in the refrigerator in an amber bottle.

ATTACHMENT 9

PROTEIN DETERMINATION

1. Add 0.1 ml of either the microsomal or cell suspensions to duplicate tubes.
2. Add 0.8 ml of water to each sample.
3. For blanks, add 0.9 ml of water.
4. To all samples and blanks, add 0.1 ml of 1% deoxycholate solution.
5. Add 4 ml of Biuret Reagent to all samples and blanks (mix well).
6. Allow samples to sit at room temperature for 20 minutes.
7. Read the absorbance (Å) versus water at 540 nm for each sample.
8. Calculate protein content as follows:

   mg protein per aliquot = (Å x 15.7)

Biuret Reagent

1.5 gm Cupric Sulfate

6.0 gm Sodium potassium tartrate

300 ml 10% NaOH

500 ml of water

Store in an amber bottle.

ATTACHMENT 10

GLYCOGEN ASSAY (ANTHRONE METHOD) PREPARATION OF REAGENTS AND ASSAY PROCEDURE

1. Anthrone Reagent

   To 100 ml of water add 400 ml of concentrated Sulfuric Acid. Cool this solution in an ice/water bath and add 0.8 grams of anthrone.

2. Procedure for Isolated Liver Cell Studies

   A. After incubation of the cell samples is completed, chill the samples in an ice/water bath. Centrifuge them at high speed for approximately 5 minutes. Separate the supernatant (to be analyzed for urea, glucose and/or other parameters) from the cell pellet in each case.

   B. a. Add 0.1 ml of 30% KOH to the cell pellet in each tube. Allow each to dissolve in the cold overnight.

      b. Place each tube in a test tube rack and cap with marbles. Place the tubes in a 80°C water bath. Heat for 15 minutes or until samples dissolve. Remove the samples from the water bath.

      c. Add 0.1 ml of water and 0.4 ml of 95% ethanol. Cap samples again with marbles and heat in a water bath (80°C) until the ethanol bubbles evolve.

      d. Chill the samples in an ice/water bath immediately. Refrigerate the samples overnight to precipitate the glycogen.

3. Centrifuge the samples (Sorvall Centrifuge at 10,000 rpm) in the cold for 30 minutes. Discard the supernatants.

4. Add an equal amount of water (amount will vary according to the glycogen content) to each tube. Shake each sample to suspend the glycogen. Take aliquots (volume may vary depending on the glycogen content) for analysis.

5. Preparation of Standards

   Solution A — To 4.9 ml of water add 100 µl of 1 mg/ml glucose solution. This solution is 20 ug/ml.

   Solution B — To 2.5 ml of solution A add 2.5 ml of water. This solution is 10 µg/ml.

   Solution C — To 2.5 ml of solution B add 2.5 ml of water. This solution is 5 µg/ml.

6. Set up 8 more tubes in the following manner:

Tube Number	Contents
1-2	1 ml of water
3-4	1 ml of solution C
5-6	1 ml of solution B
7-8	1 ml of soluiton A

7. To tubes 1 through 8 and each sample, add 5 ml of anthrone reagent. Keep the samples cold while adding the reagent.

8. Shake well and put these tubes in a boiling water bath for 8 minutes using marbles to prevent evaporation.

9. Remove the samples from the boiling water bath and place them in an ice/water bath. The resulting solutions should be green.

10. Determine the absorbance of each sample at 625 nm with the aid of a spectrophotometer.

11. Calculate the amount of glycogen in glucose equivalents for each sample using the results obtained from the standards.

Note: The anthrone method for glycogen was adapted from Mortimore, G.E.; King, E.,Jr.; Mondon, C.E.; and Glinsman, W.H. 1967. Effects of Insulin on Net Carbohydrate Alterations in Perfused Rat Liver. Am. J. Physiol. 212:179

APPENDIX 4

SGRD-UE-HR 1 August 1986

MEMORANDUM FOR: SEE DISTRIBUTION

SUBJECT: Action of USARIEM Laboratory Animal Care and Use Committee re: Modification and resubmission of the proposal entitled "The effects of cortisol, insulin and glucose treatment on heatstroke in rats" submitted by Dr. Margaret E. Tolbert, Carver Research Foundation

1. The original protocol entitled "The effects of cortisol, insulin and glucose treatment on heatstroke in rats" was considered initially at the 12 May 1986 meeting of the USARIEM LACUC chaired by (then) CPT G. R. Silver, D.V.M. The minutes of this meeting indicated that members of the committee recommended extensive modification of the protocol and resubmission to the LACUC after suitable revision. The recommendations for revision were detailed in the minutes of the 12 May 1986 meeting of the LACUC.

2. The protocol was returned to Dr. Tolbert and in a letter to COL Joyce with 10 attachments dated 11 July 1986, Dr. Tolbert detailed the revisions to the protocol which she had elected to make to respond to the suggestions of the USARIEM LACUC.

3. During that interval CPT Silver was replaced as Chairman, LACUC by the undersigned.

4. On 21 July 1986 I received a call from Dr. Tolbert regarding the progress on her protocol by the USARIEM, LACUC. Unfortunately, I was not on the distribution list for Dr. Tolbert's 11 July letter and had not seen it so I was unable to report any progress. However, on that same day (21 July) I obtained a copy of Dr. Tolbert's letter containing the revisions to the protocol, and on 22 July 1986 distributed a copy of that letter to all members of the LACUC who voted on the original submission. Thus, members of the USARIEM LACUC have now evaluated the revisions to the original protocol outlined in Dr. Tolbert's letter.

5. Dr. Tolbert's proposal is generally in three parts and several committee members elected to consider each portion separately.

a. Cortisol, insulin, and glucose as a post-heating therapeutic regimen.
The LACUC agreed that the modifications which Dr. Tolbert submitted were entirely adequate as written and no further alterations are necessary on this portion.

b. Kinetics of hormone uptake in normal and hyperthermic animals
Although the majority of the LACUC recommended approval of this substudy as revised by Dr. Tolbert, the following additional recommendations are submitted for consideration. It is possible that the same rat can be sampled for 60 min. At least 2 members of the LACUC believe that with the

SGRD-UE-HR 1 August 1986
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radiolabelled tracer to be used, a blood volume of 0.1 ml may provide sufficient volume for counting. If this is the case, then the kinetic studies will be less variable (4 time points on a single rat rather than on 4 separate rats should provide more consistent kinetic data) and fewer rats will be necessary for comparison of normothermic and hyperthermic animals. A further recommendation for consideration is the use of a chronically implanted cannula in the jugular vein for label injection to replace the currently proposed femoral vein injection. The committee recommends extreme care in the assay of the other variables (LDH, K^+, CPK, SGOT) using radioactively contaminated plasma.

c. Response of isolated hepatocytes to heat and hormones.

Although the majority of the LACUC recommended approval of this substudy as revised by Dr. Tolbert, the following additional comments are submitted for consideration. At least two members of the LACUC believe that if sufficient numbers of viable hepatocytes can be isolated from a single rat for experimental purposes, then the total number of rats currently requested (100) may be significantly reduced.

6. In summary, the *USARIEM LACUC recommends* approval of all three parts of the protocol as amended by the letter of 11 July 1986 signed by Dr. Tolbert. The USARIEM LACUC also requests the principal investigator to consider the recommendations noted in #5 b,c above.

RALPH P. FRANCESCONI, Ph.D
Interim Chairman, USARIEM
Laboratory Animal Care & Use Committee

APPROVAL/D[redacted]L
DAVID D. SCHNAKENBERG
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