EFFECTS OF COLD STRESS ON CELLULAR STRUCTURE AND FUNCTION

RALPH BUCHSBAUM
MONTE BUCHSBAUM
THOMAS LINSENMAYER

UNIVERSITY OF PITTSBURGH

FOREWORD

This investigation was initiated by the Biothermal Branch, Environmental Medicine Division, Biomedical Laboratory, Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio. The research was conducted by the Department of Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, under Contract No. AF 33(615)-1076. The work was performed under Project 7164, "Biomedical Criteria for Aerospace Flight," Task 716409, "Human Thermal Stress." This work is an extension of that reported by Buchsbaum, Ralph, Thermal Stress on Cellular Structure and Function, Aerospace Medical Research Laboratories Technical Documentary Report No. AMRL-TDR-63-14 (AD 402 905), Wright-Patterson Air Force Base, Ohio, February 1963. Mr. J. F. Hall, Jr, was contract monitor for the Aerospace Medical Research Laboratories. The study began in November 1963 and was completed in November 1965.

This technical report has been reviewed and is approved.

J. W. HEIM, PhD
Technical Director
Biomedical Laboratory
Aerospace Medical Research Laboratories

ABSTRACT

It is well known that cells in vitro can be quick-frozen (with the aid of glycerol in the medium) to -70 C, stored for months, and after being warmed to 37 C, continue to live normally. Yet cells taken slowly to a few degrees below freezing commonly die. The purpose of this investigation was to ascertain more precisely the limits of tolerance of cells in vitro to cold, as a basis for investigations on the cellular mechanisms affected. Mouse kidney cells were maintained in a perfusion chamber arranged for cooling and warming at various rates. Normal tissue culture media were used. Nearly all cells exposed to -0.3 C for 10 minutes, then warmed to 37 C, lived. Nearly all cells exposed to -1 C or lower for 10 minutes, and warmed to 37 C, died. The critical lethal cold shock seems to be between -0.3 and -0.7 C. Cold shock seems to produce little obvious change in cells, but mitochondrial breaks and swelling are obvious in electron micrographs. Also, nuclear ground substance appeared more granular than normal. One hypothesis was that microcrystallization probably occurs in certain organelles, particularly the mitochondria because these organelles are in constant motion. Similar results were obtained in experiments with human neutrophiles.

SECTION I

INTRODUCTION

This study is an extension of that reported in Ref. 1. It is concerned with the effects of exposing living mammalian cells in tissue culture chambers to various periods of abnormally high or low temperatures.

In the previous report, mouse kidney cells were grown on coverslips in a perfusion chamber which permitted continuous change of medium, microscopic examination, photography, and precise temperature control and measurement. Cells were exposed to normal body temperature for a time and then to an elevated temperature for a period of 1 to 10 minutes. The temperature was then returned to normal. Generally, these cells appeared normal up to 42 C. The maximum temperature to which cells were exposed was found to be more important as a factor in determining survival than the duration of exposure to elevated temperature. No cells survived treatments at 53 C for period of 1 to 10 minutes. A small proportion of cells survived heat-shocks of 50 and 47 C, with less survival at 50 than at 47 C, as would be expected.

Observation of the cells revealed a standard pattern of response. A series of five stages in the thermal cytosyndrome was established, which makes it convenient to refer to the degree of cellular damage encountered in a heat shock under the conditions of the experiment. It would be interesting to see whether these stages can be recognized in cases of burns in vivo.

In the experiments reported here, most of the effort went into the study of cells exposed to low temperatures. Most of the literature on the effect of exposing cells in vitro to low temperatures deals with the preservation of cells at extremely low temperatures for the purpose of maintaining strains of cells inactive for future study. Our purpose was to ascertain the limits of tolerance of cells to cold, as a basis for investigations on the cellular mechanisms affected.

SECTION II

MATERIAL AND METHODS

1. Perfusion chamber. Various types of cell preparations were made from kidneys removed from laboratory mice by the methods described in Ref. 1. In some of the cold experiments, cells were maintained in a perfusion chamber shown in Fig. 1. This was especially designed for these experiments. It consisted of a flat cylindrical chamber between two coverslips held apart by a silicone rubber ring. The coverslips and ring were held in a stainless steel assembly. The ring was perforated by three needles, two of which carried a flow of nutritive medium through the chamber, and one contained a a thermistor probe which enabled us to control and record the temperature in the chamber. The perfusion chamber was mounted in a recess in a stainless steel slide which

Ref. 1. Technical Documentary Report No. AMRL-TDR-63-14, February 1963.

was placed on the stage of the microscope as shown in Fig. 1. Cooling was accomplished

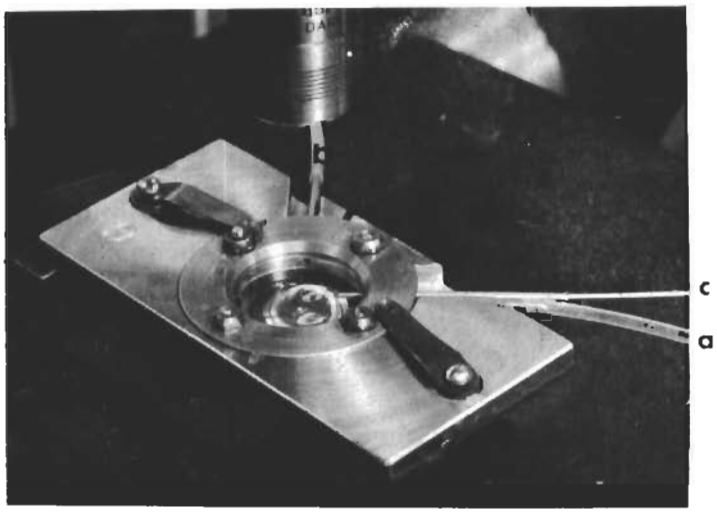


Fig. 1. Perfusion chamber, showing three leads: a, inflow; b, outflow; c, thermistor probe.

by pumping cooled nutritive medium through the chamber. However, to cool the chamber, rapidly and not disturb the optimum flow of medium through the chamber, an alternate method was devised. The stainless steel perfusion chamber holder was replaced

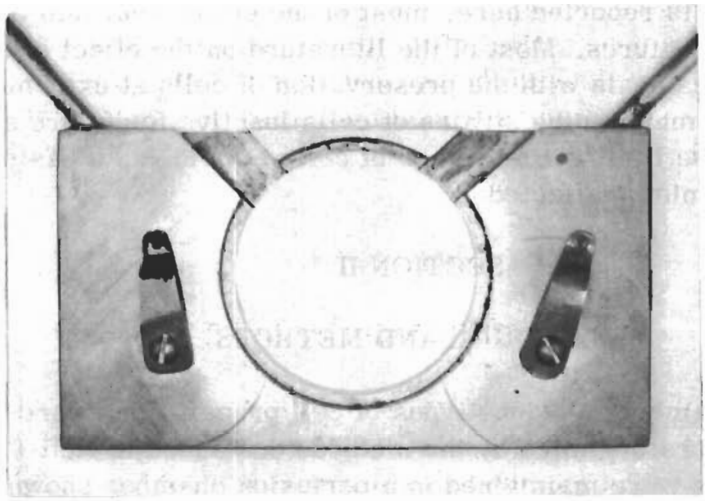


Fig. 2. Brass holder for perfusion chamber. The cooling fluid travels in channels in the brass. Natural size.

by a brass slide (fig. 2) constructed with a double wall to allow a coolant fluid to flow in channels running in both sides. Cooling the slide resulted in cooling the chamber by conduction. Fig. 3 shows a preparation on the stage of the microscope. Note the frost on the brass slide and adjacent tubing. The temperature of the cooling fluid pumped from a dewar flask was -70 C; this permitted a very rapid rate of cooling in the per-

fusion chamber. A second dewar flask contained ethanol at 47 C for rapid warming following the cooling. The rate of cooling (or heating) was controlled by a proportional timer as shown in Fig. 4. This activated the pumping of the cold (or hot) alcohol. After a preparation was exposed, say to 0 C for 10 minutes, the cold alcohol was shunted off

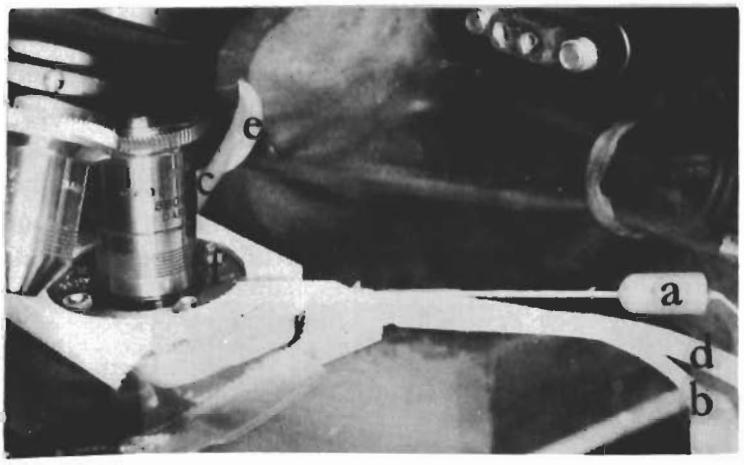


Fig. 3. Preparation on stage of microscope. a, thermister probe; b, c, perfusion lines carrying medium; d, e, lines carrying cold alcohol through brass holder; f, perfusion chamber.

(by pinch clamps on the hoses) and other branches of two Y-tubes were opened to allow warm alcohol to flow through the brass holder instead. Warming to, say, 37 C was programmed to occur at various rates, depending upon the experiment the investigator de-

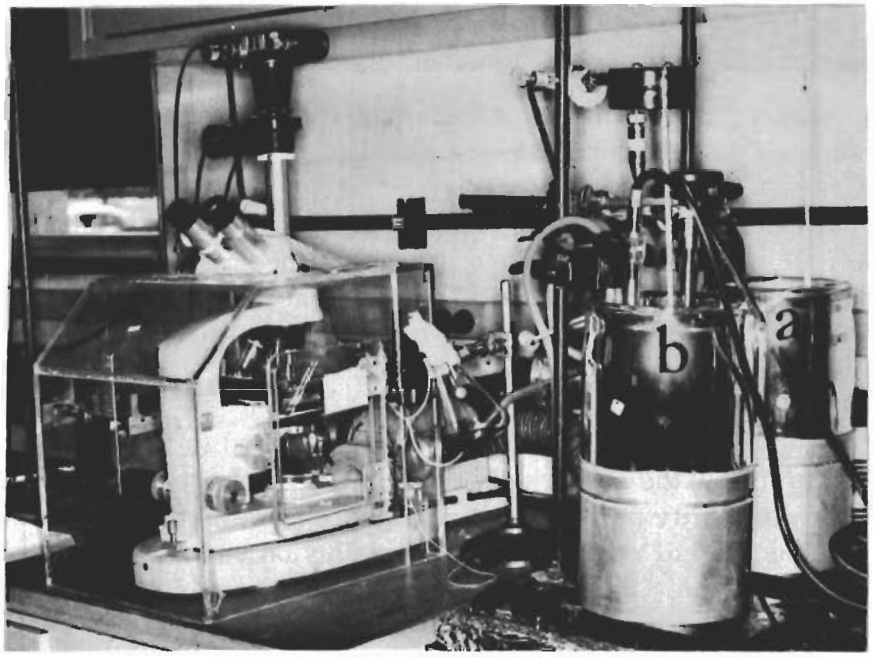


Fig. 4. Microscope and dewar flask assembly. a, cold alcohol bath at -70 C with toluene thermometer; b, hot alcohol bath with stirrer, heater, and thermostat.

sired by regulating the pumping rate. This was accomplished by regulating the proportional timer. A typical cold-stress experiment was made by observing and photographing cells in the perfusion chamber before, during, and after the temperature excursion. Runs were made to various temperatures from 0 to -7 C. Cells were observed for about 48 hours after exposure to the low temperature to be sure that those recovering had an adequate chance to do so.

2. Blood cells. In a second group of experiments blood was drawn from volunteers into a 30 ml syringe wetted with a 1:5000 heparin solution. After centrifuging, the buffy coat was drawn off with some plasma. The 0.5 ml aliquots of the cell suspension were distributed into Wasserman tubes. The tubes were then suspended in alcohol baths at various temperatures between 0 and -13 C. Recordings with a thermistor probe in a tube showed that the contents reached within 1 C of the bath temperature in less than 30 seconds. After

Fig. 5. Two 1x3" slides plunged into -4.0 C alcohol-ice bath. The slides are contained in an evacuated polyethylene bag.

a 10 minute cold treatment, the tubes were warmed to 37 C in a hot air incubator for 2 hours. Drops of the cell suspension were then put onto slides, covered with a coverglass, sealed with Vaseline® and examined with the oil immersion lens of a phase-contrast microscope. The polymorphonuclear leucocytes (neutrophils) were counted for survival. The criterion for life was ameboid motion.

3. Immersion of whole cultures. A third method used proved to be less elegant in that we were not able to follow the same individual cell throughout the observation, but it had the advantage of simplicity so that larger numbers of cells could be observed in a given experiment. It consisted of merely placing a perfusion chamber or coverslip mounted on a hollow-ground slide in a polyethylene bag and immersing it in the cooling bath (fig. 5) at a known temperature—kept to within 0.01 C for as long as desired; 10 minutes was the arbitrary time selected. Then the chamber was removed, placed on the stage of the microscope in a hot air bath and observed. A variety of temperatures between 0 and -0.7 C were used for reasons explained below.

4. Electron micrographs. A fourth method of study consisted of exposing cells to supercooling, bringing them back to normal, fixing, embedding, sectioning, and making electron micrographs. Controls were similarly prepared of uncooled cells.

SECTION III

RESULTS AND CONCLUSIONS

1. Nearly all cells cooled to 0C, kept at this temperature for 10 minutes, and warmed to 37 C, survived for at least 48 hours and showed no damage. Nearly all cells exposed to -1.0 C or lower for 10 minutes, and warmed to 37 C, died. Most of the preparations cooled to -.3 C survived. The critical lethal shock seems to be between -0.3 and -0.7 C. As the temperature fell from 0 C, surprising little change was seen. There was a change in the appearance of the nuclear ground substance; it became more granular and somewhat less translucent. And the most conspicuous change was in the transverse breaking of the longer mitochondria. Some mitochondria were swollen. Commonly cells became less adherent to the coverslip and appeared more rounded. Some of these changes are seen in figs. 6, 7, 8, and 9. The typical mitochondrial movement, though slower, continued in cells cooled to -9.0 C. The freezing point of the medium is approximately -0.67 C. But only occasionally did crystallization occur. Indeed, it was relatively difficult to induce crystallization. This was fortunate, for when it did occur, as happened a few times, the expanding ice smashed the coverslip, and the experiment was lost.

The problem of ascertaining the moment of cell death is a difficult one and there is an extensive literature on this point. Except for "violent" death due to protein denaturation, as produced by heat, toxins, etc, cell death is most evident by cessation of normal movements of the mitochondria, cessation of cell membrane movement, withdrawal of cell processes, and precipitation of normally clear or very faintly granular areas of cytoplasm. Many other processes occur in injured cells, such as the formation of vacuoles, but many of these processes are reversible. It is our hypothesis that microcrystallization may occur in certain cell organelles and produce irreversible destruction on a submicroscopic level which results in cell death. The fact that mitochondria are in constant motion is evidence that they may provide the site for such crystallization.

2. Blood cells. Heterophil granulocytes survived cooling to 0 C as above. The curve below represents data from two human subjects and 600 to 1000 cells for each point. Standard probit analysis techniques were used to fit survival curves. The 50% survival rate occurred at -7.0 C. Incidentally, the heat-stress survival curve resembled the cold-stress curve rather closely with the 50% point at 45 C.

With a little care, cell suspensions have been kept at -13 C for 30 minutes without crystallization, but since practically all cells died after exposure to -9 C there was no point in using lower temperatures.

Experiments were made in which three tubes of blood cells were exposed to the sequences of temperatures shown in table I.

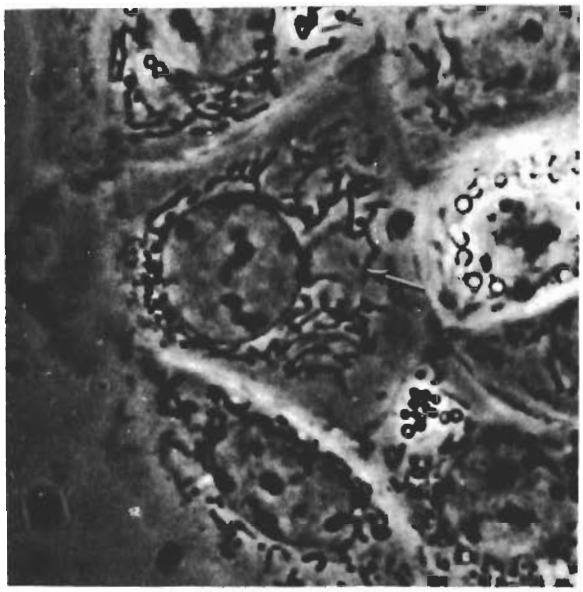


Fig. 6. Normal mouse kidney epithelial cell in depression slide chamber. Mitochondria (arrows) normal, rodlike. Phase contrast microscopy.

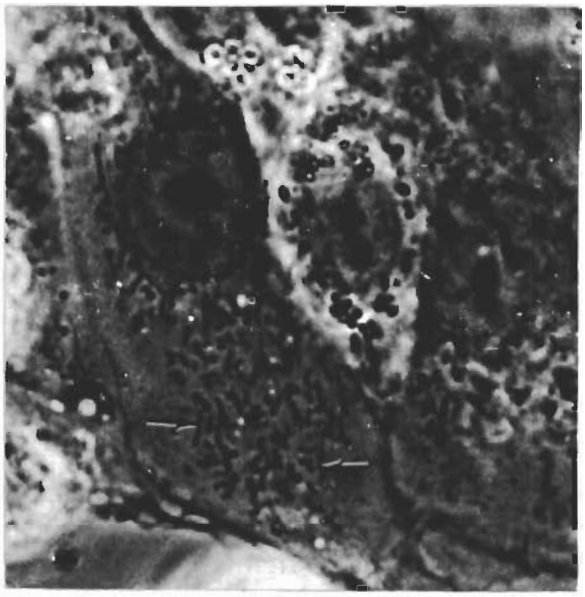


Fig. 7. Similar preparation, 30 min after cooling by immersion procedure. Mitochondria (arrows) fragmented, few are normal.

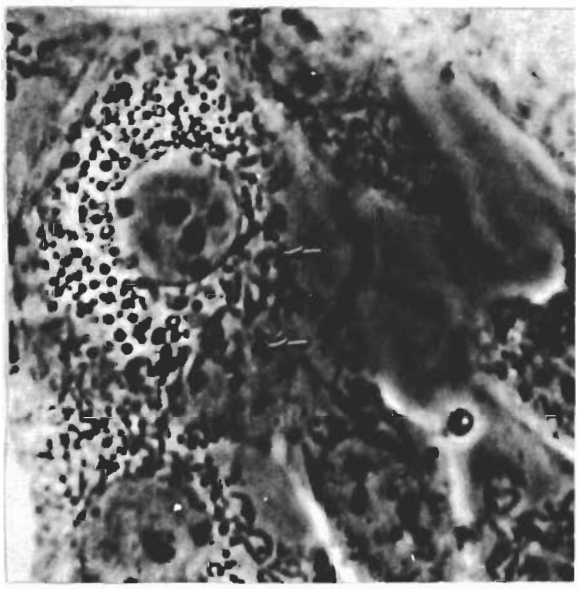


Fig. 8. Same preparation, 1 hour after cooling by immersion procedure. Mitochondria (arrows) swollen. In some cells mitochondria are less affected than in others. Vacuoles increase in number.

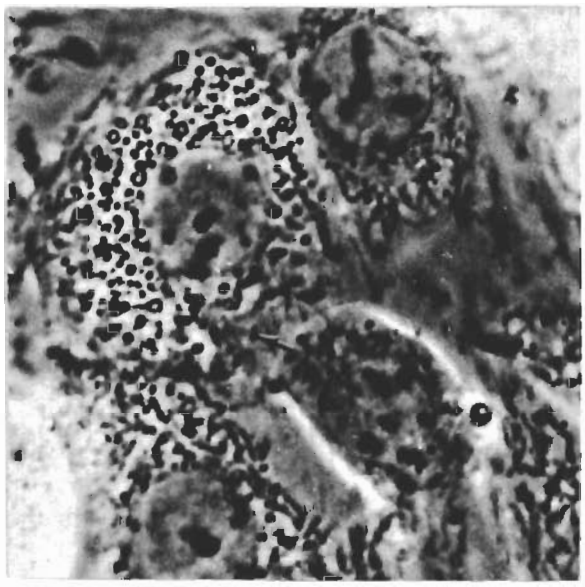
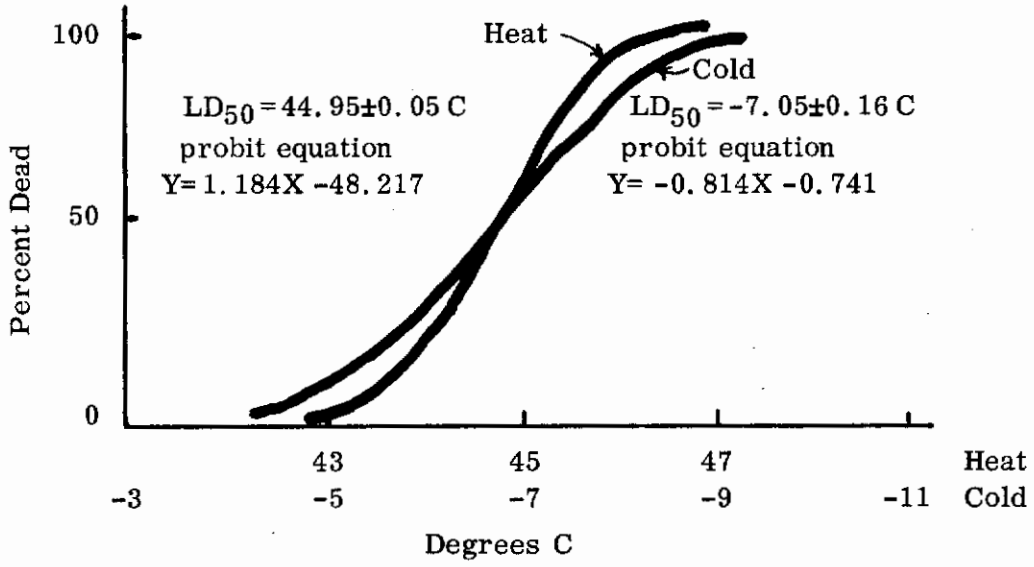


Fig. 9. Same preparation, 1.5 hours after cooling by immersion procedure. Mitochondria (arrows) fewer and the vacuolar area is increased. Some shrinkage of cytoplasm.

Table I

Tube A	Tube B	Tube C
30 min at 0 C	30 min at 37 C	30 min at 37 C
10 45	10 45	10 45
180 37	30 0	180 37
	180 37	
Results: 76.2% survived	47.0% survived	52.0% survived

Differences between pretreatment with cold and no pretreatment were statistically significant in every replication of the experiment. Pooled results from these three experiments with 500 cells counted in each type of treatment are significant at greater than the 1% level. White blood cells supercooled to -3 C were somewhat



protected from damage at 45 C, but not as protected as cells pretreated by cooling to 0C. The differences in cell survival in tubes pretreated by cooling to -3 C and without pretreatment and pretreatment at 0C were statistically significant ($p < 0.05$).

3. Experiments with treatment by immersion of cell preparations in perfusion chambers in polyethylene bags, in an alcohol bath, were consistent with the results of the above described observations. Table II shows some of the results.

In the neighborhood of -0.67 C some irreversible change seems to occur, despite the apparent absence of macroscopic or microscopic crystallization or any obvious change in the cell at the time of cooling or immediate restoration to normal temperature.

4. Electron micrographs. Cells were exposed to supercooling, fixed, embedded, sectioned, stained, and electron micrographs made. Controls were similarly pre-

Table II

100% Survival after 48 hours	100% Death after 48 hours
-0.35±0.01 C	-0.67±0.01 C
.40	.70
.60	.80
.65	1.00
.66	1.00
.66	1.00

pared. The controls (fig. 10) show the normal mitochondria. The cooled preparations (fig. 11) were taken down to -4.0C for 20 minutes then returned to 37 C for 90 minutes before being fixed. Note the enormous change in the mitochondria; there seems to be little damage elsewhere in the cell.

SECTION IV

DISCUSSION

The purpose of this research and that of the previous study (Ref. 1) is to investigate the behavior of cells with respect to heat and cold shocks: morphology and function. In respect to morphology the picture is becoming fairly clear, in so far as we can generalize from the experiments. At deviant high temperatures cells exhibit a standard heat cytosyndrome that is a continuous process, but, like mitosis, can be described in stages (Ref. 1). The cold cytosyndrome produces no such stages, but certain disruptions occur at the freezing point of certain cell fluids. In the absence of overt crystallization, the damage is probably due to the formation of ultramicroscopic ice crystals that rupture membranes such as those associated with the mitochondria.

An attack on the functional aspects is more difficult. On the tentative hypothesis of differentially temperature-sensitive enzyme pathways, it may be that substrate or product concentration in any step may be deficient and so block a given metabolic pathway. On the high temperature side, such pathways may be irreversibly destroyed so that cell death ensues. On the cold side, such paths probably are more frequently merely blocked and recover when the temperature is returned to normal, except, of course, if the low temperature distrupts the structure permanently.

To establish a more detailed explanation requires a new approach. We propose to use radioactive tracers to try to identify the particular mechanisms involved. For example, there may be cold blocks to certain enzyme pathways as the temperature is lowered and at the critical temperature region about -0.7 C, an additional factor of destruction of surfaces or other structures on which such enzymes might act. It is likely that even though mitochondria in a cold-shocked cell returned to 37 C may not be functioning, the cell can survive for some hours, coasting on processes (e.g., the production of ATP) begun before they ceased functioning.

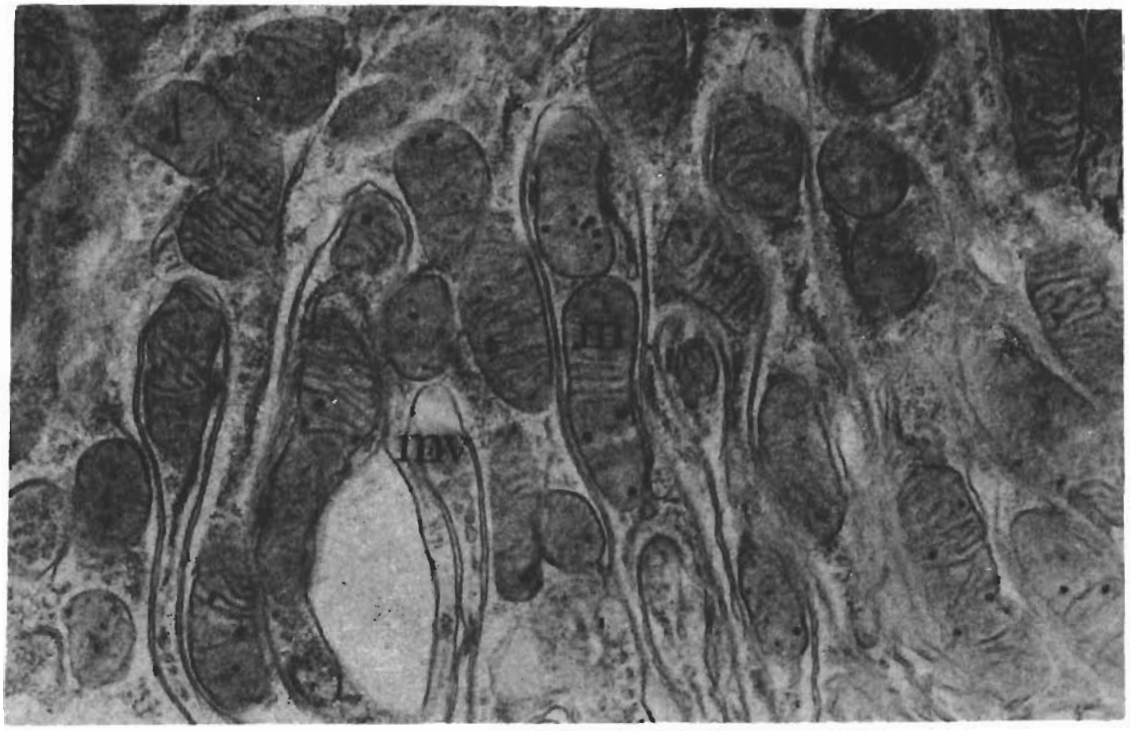


Fig. 10. Electron micrograph of normal mouse kidney cell. Fixed in phosphate-buffered oxmium tetroxide and lead stained (method of Dalton and Zeigel). m, mitochondrion; n, nucleus; mv, microvilli of brush border; l, lysosome.

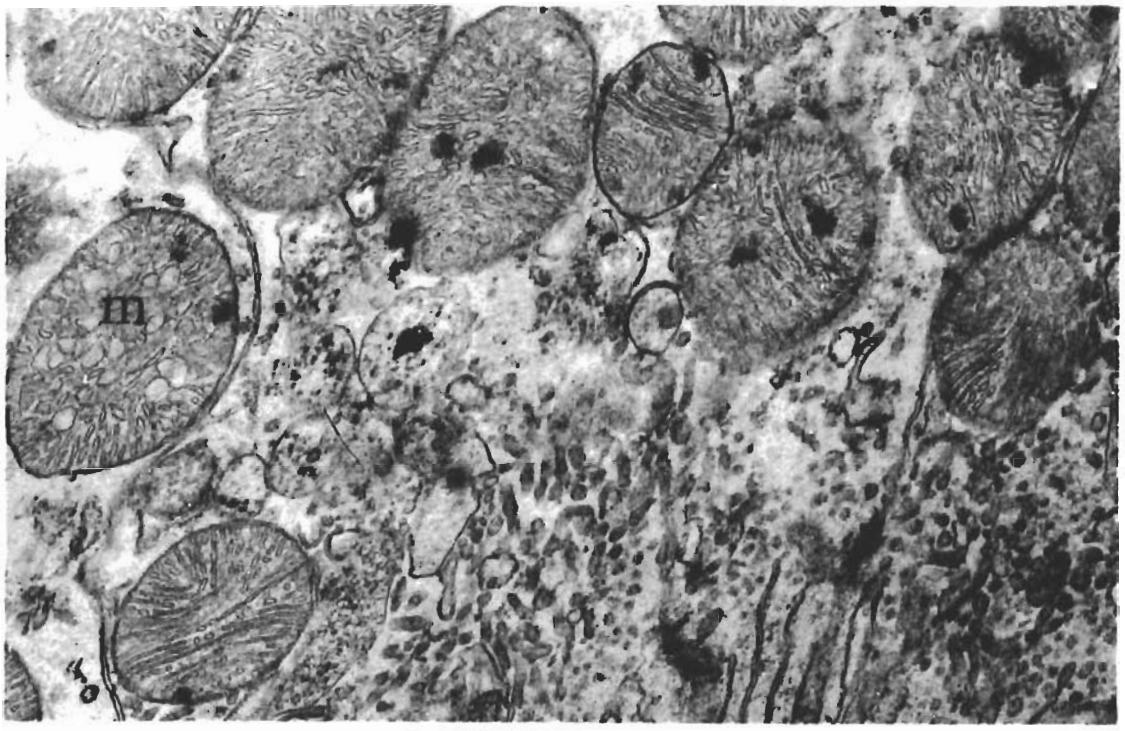


Fig. 11. Electron micrograph of mouse kidney cell supercooled at -4.0 C for 20 min., incubated at 37 C for 90 min., then fixed as above. Note unusual appearance of mitochondria: enlarged and with vacuolar structures.

Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
University of Pittsburgh
Department of Biology
Pittsburgh, Pennsylvania 15213

2a. REPORT SECURITY CLASSIFICATION
UNCLASSIFIED

2b. GROUP
N/A

3. REPORT TITLE

EFFECTS OF COLD STRESS ON CELLULAR STRUCTURE AND FUNCTION

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Final report, November 1963 - November 1965

5. AUTHOR(S) (Last name, first name, initial)
Buchsbaum, Ralph
Buchsbaum, Monte
Linsenmayer, Thomas

6. REPORT DATE
May 1966

7a. TOTAL NO. OF PAGES
14

7b. NO. OF REFS
1

8a. CONTRACT OR GRANT NO. AF 33(615)-1076

b. PROJECT NO. 7164

c. Task No. 716409

d.

9a. ORIGINATOR'S REPORT NUMBER(S)

9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
AMRL-TR-66-30

10. AVAILABILITY/LIMITATION NOTICES

Distribution of this document is unlimited.

11. SUPPLEMENTARY NOTES

12. SPONSORING MILITARY ACTIVITY
Aerospace Medical Research Laboratories, Aerospace Medical Div., Air Force Systems Command, Wright-Patterson AFB, Ohio

13. ABSTRACT

It is well known that cells in vitro can be quick-frozen (with the aid of glycerol in the medium) to -70 C, stored for months, and after being warmed to 37 C, continue to live normally. Yet cells taken slowly to a few degrees below freezing commonly die. The purpose of this investigation was to ascertain more precisely the limits of tolerance of cells in vitro to cold, as a basis for investigations on the cellular mechanisms affected. Mouse kidney cells were maintained in a perfusion chamber arranged for cooling and warming at various rates. Normal tissue culture media were used. Nearly all cells exposed to -0.3 C for 10 minutes, then warmed to 37 C, lived. Nearly all cells exposed to -1 C or lower for 10 minutes, and warmed to 37 C, died. The critical lethal cold shock seems to be between -0.3 and -0.7 C. Cold shock seems to produce little obvious change in cells, but mitochondrial breaks and swelling are obivous in electron micrographs. Also, nuclear ground substance appeared more granular than normal. One hypothesis was that microcrystallization probably occurs in certain organelles, particularly the mitochondria because these organelles are in constant motion. Similar results were obtained in experiments with human neutrophiles.

DD FORM 1 JAN 64 1473

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Tolerance limits						
Cellular mechanisms						
Cold shock						
Heat shock						
Electron microscopy						
Morphology						
Cytology, experimental						
Mice kidney cells						
Human blood cells						

INSTRUCTIONS

1. ORIGINATING ACTIVITY: Enter the name and address of the contractor, subcontractor, grantee, Department of Defense activity or other organization *(corporate author)* issuing the report.

2*a*. REPORT SECURITY CLASSIFICATION: Enter the overall security classification of the report. Indicate whether "Restricted Data" is included. Marking is to be in accordance with appropriate security regulations.

2*b*. GROUP: Automatic downgrading is specified in DoD Directive 5200.10 and Armed Forces Industrial Manual. Enter the group number. Also, when applicable, show that optional markings have been used for Group 3 and Group 4 as authorized.

3. REPORT TITLE: Enter the complete report title in all capital letters. Titles in all cases should be unclassified. If a meaningful title cannot be selected without classification, show title classification in all capitals in parenthesis immediately following the title.

4. DESCRIPTIVE NOTES: If appropriate, enter the type of report, e.g., interim, progress, summary, annual, or final. Give the inclusive dates when a specific reporting period is covered.

5. AUTHOR(S): Enter the name(s) of author(s) as shown on or in the report. Enter last name, first name, middle initial. If military, show rank and branch of service. The name of the principal author is an absolute minimum requirement.

6. REPORT DATE: Enter the date of the report as day, month, year; or month, year. If more than one date appears on the report, use date of publication.

7*a*. TOTAL NUMBER OF PAGES: The total page count should follow normal pagination procedures, i.e., enter the number of pages containing information.

7*b*. NUMBER OF REFERENCES: Enter the total number of references cited in the report.

8*a*. CONTRACT OR GRANT NUMBER: If appropriate, enter the applicable number of the contract or grant under which the report was written.

8*b*, 8*c*, & 8*d*. PROJECT NUMBER: Enter the appropriate military department identification, such as project number, subproject number, system numbers, task number, etc.

9*a*. ORIGINATOR'S REPORT NUMBER(S): Enter the official report number by which the document will be identified and controlled by the originating activity. This number must be unique to this report.

9*b*. OTHER REPORT NUMBER(S): If the report has been assigned any other report numbers *(either by the originator or by the sponsor)*, also enter this number(s).

10. AVAILABILITY/LIMITATION NOTICES: Enter any limitations on further dissemination of the report, other than those imposed by security classification, using standard statements such as:

(1) "Qualified requesters may obtain copies of this report from DDC."

(2) "Foreign announcement and dissemination of this report by DDC is not authorized."

(3) "U. S. Government agencies may obtain copies of this report directly from DDC. Other qualified DDC users shall request through _____."

(4) "U. S. military agencies may obtain copies of this report directly from DDC. Other qualified users shall request through _____."

(5) "All distribution of this report is controlled. Qualified DDC users shall request through _____."

If the report has been furnished to the Office of Technical Services, Department of Commerce, for sale to the public, indicate this fact and enter the price, if known.

11. SUPPLEMENTARY NOTES: Use for additional explanatory notes.

12. SPONSORING MILITARY ACTIVITY: Enter the name of the departmental project office or laboratory sponsoring *(paying for)* the research and development. Include address.

13. ABSTRACT: Enter an abstract giving a brief and factual summary of the document indicative of the report, even though it may also appear elsewhere in the body of the technical report. If additional space is required, a continuation sheet shall be attached.

It is highly desirable that the abstract of classified reports be unclassified. Each paragraph of the abstract shall end with an indication of the military security classification of the information in the paragraph, represented as *(TS)*, *(S)*, *(C)*, *or (U)*.

There is no limitation on the length of the abstract. However, the suggested length is from 150 to 225 words.

14. KEY WORDS: Key words are technically meaningful terms or short phrases that characterize a report and may be used as index entries for cataloging the report. Key words must be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location, may be used as key words but will be followed by an indication of technical context. The assignment of links, rules, and weights is optional.