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TYPES OF FREEZING AND THE POST-THAWING SURVIVAL OF MAMMALIAN TISSUE

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Akira HŌKI. *Types of Freezing and the Post-thawing Survival of Mammalian Tissue.* Kobe J. Med. Sci. **13**, 67-79, March 1967——The cryoprotective effect of dextran, glycerol and dimethyl sulfoxide was compared on kidney tissue. The author perfused cat's kidney *in vivo* with cold artificial solution and administered cryoprotective additives through vascular system. Tangential slices of the kidney were frozen and thawed under various possibly regulated rates of freezing and thawing, and the effect of cryoprotective agents were evaluated by oxygen uptake and photomicrographs of fixed and stained tissue. The results obtained showed the least damage in the tissue at slow rate of freezing and thawing. Dextran showed cryoprotective effect only in case of slow rate. With regard to dimethyl sulfoxide high protective effect was observed on an average for slow, rapid and ultrarapid freezing and thawing. Glycerol showed, in general, marked protective effect especially at slow and fast rate treatment.

INTRODUCTION

Recently, with the advance of research for transplantation of organs or tissues, it has been paid much attention to the preservation method. Since freezing was adopted as one method of the preservation of living materials, many attempts have been carried to protect the injury caused by freezing. By this time, preservation by freezing of particular cells or the isolated cells such as red blood cells and cultured single cells has been achieved with success by many authors[1)5)8)9)12)16)18)] and they presented a definite mode of freezing and thawing, i. e. ultrarapid freezing to and thawing from very low temperature with the support of cryoprotective additives. The freezing experiment with organs[18)20)] and tissues[18)17)19)], however, is rather few. Moreover, physical and biological conditions in freezing and thawing are not so much exactly quantitative that the same results are hardly obtained repeatedly by others. In addition, the relative effect of the used cryoprotective agents was not compared each other conclusively. Since Beltran & Blumenthal[8)] first reported the frozen experiment of kidney (1951), kidney has examined from many aspects, i. e. procedure of perfusion, way to apply the cryoprotective agents and rate of freezing and thawing have been studied. But being due to its relative large volume,[14)] it was difficult to freeze and thaw the every cellular component of the kidney with a definite rate of temperature changes and to compare the resistance of the component cells to freezing under constant condition. Moreover, it has not been done any available comparison between the effects of the cryoprotective

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agents.

The author perfused cat's kidney *in vivo* with cold artificial solution to replace the blood with it and administered cryoprotective additives which had been dissolved in Hanks solution through the vascular system to secure full distribution. Tangential slices of the kidney which involves the cortex and medulla were frozen and thawed under various possibly regulated rates of freezing and thawing, and the effects of cryoprotective agents were evaluated with oxygen uptake and photomicrographs of the sliced tissue.

METHOD

Adult cat's weighing 2-3 kg were anesthetized with Nembutal intraperitoneally and administered 5 mg/kg heparin intravenously and the kidney of the cat was used for this experiment. As a preliminary stage, one of the kidneys was perfused[20] pulsatory through the renal artery with 4% dextran Hanks solution at 5°C *in situ* in order to replace blood by the artificial fluid. Immediately preceding perfusion ligature and cutting were made on as much as possible branches of renal vessels; such as the inferior suprarenal branches of renal artery, the left suprarenal vein, the left spermatic or ovarian vein and other vessels and massligature was also placed on the surrounding adipose tissues. Pulsation pressure was 90/70 mmHg and pulsation rate was 120/min. Unperfused kidney of the other side served as control and the perfused kidney was compared with it.

Hanks solution supplemented dextran to 4% (mol. wt. 63,000) was used as the basic perfusate and glycerol or dimethyl sulfoxide (DMSO) was added in it as cryoprotective additives. By *in vivo* preliminary perfusion with dextran Hanks solution for 30 to 40 minutes, blood was usually replaced completely by the perfusate. The perfusion was continued successively with gradual increasing concentration of glycerol or DMSO, first with 5 ml/dl of glycerol or DMSO solution for 20 minutes, then 10 ml/dl of the solution for 10 minutes, and finally with 15 ml/dl of the solution for 20 minutes.

To compare the cryoprotective effects of dextran, DMSO and glycerol the kidney was extirpated immediately after perfusion ceased. Wedge-shaped slices which involved cortex and medulla of the kidney weighing 10 to 40 mg with a thickness of about 300μ were prepared for freezing.

Three grades of the rate of freezing were established in this experiment; as slow freezing (s-f) 2°C lowering per minute, as rapid freezing (r-f) 25°C per minute and as ultrarapid freezing (u-f) 800°C per minute. For slow or rapid freezing slices were frozen directly on the plate of the heat-exchange apparatus and the temperature of the tissue was lowered to −8°C from +5°C in the case of control and dextran slices and brought down to −12°C from +5°C in the case of the tissue perfused with glycerol and DMSO. For ultrarapid freezing slices put between two sheets of aluminum foil were immersed in aceton-dry ice mixture and the temperature turned from +5°C to −79°C. All the slices were left for one minute at each extreme temperature and thawing procedure was performed.

The rate of thawing was also controlled with three grades; as slow thawing

(s-t) 2°C raising per minute, as rapid thawing (r-t) 45°C~55°C per minute and as ultrarapid thawing (u t) 1000°C per minute. For slow or rapid thawing slices were thawed also on the plate of the heat-exchange apparatus and the temperature turned from −8°C or −12°C to +3°C or +15°C. For ultrarapid thawing aluminum foil holding slices in aceton-dry ice mixture was dipped into a water bath (37°C) immediately (Table 1).

Table 1. Conditions of freezing and thawing used in this experiment.

	CLASSIFICATION	METHOD	RATE OF FREEZING & THAWING (°C/min)	TEMPERATURE RANGE CONTROLLED D-H(°C)	TEMPERATURE RANGE CONTROLLED G-D-H, DMSO D-H(°C)
FREEZING	Slow	Electro-thermo-element	2	+5→ −8	+5→−12
	Rapid		25	+5→ −8	+5→−12
	Ultrarapid	Acetone-dry ice	800	+5→−79	+5→−79
THAWING	Slow	Electro-thermo-element	2	−8→+ 3	−12→+ 3
	Rapid		45~55	−8→+15	−12→+15
	Ultrarapid	Water bath	1000	−79→+37	−79→+37

D-H : Dextran Hanks solution
G-D-H : Glycerol Dextran Hanks solution

The effect of cryoprotective additives was evaluated by oxygen consumption and histological examination.

For histological examination slices after freezing and thawing were fixed with 10% formalin and histological sections (6 or 12μ) were prepared by freezing and stained with hematoxylin eosin. On measurement of oxygen consumption by tissue slices Warburg's manometric method was employed. Two ml Krebs-Ringer-phosphate solution was pipetted in the main compartment and gas absorption reagent (0.2 ml 5% KOH paper) was introduced into the center well. Air in gas phase was replaced by pure gas and medium was saturated with pure oxygen. Thermostat was brought to 37°C and shaking was a reciprocating motion in a horizontal plane at the rate of 100 to 110 excursions of 5 cm in each direction per minute. The reading was carried out for 1 hour. Then the weight of slices was measured. The tissue respiration was calculated at the rate of μl O_2/mg wet weight/hr.

RESULTS

Effects of the perfusate upon the tissue respiration.

Before evaluation of the protective action of cryoprotective agents, biological effects of *in vivo* perfusion with the artificial fluid involving cryoprotective agents were examined. For the purpose, oxygen uptake of sliced kidney after perfusion

Table 2. EFFECT OF IN VIVO PERFUSION ON TISSUE RESPIRATION

μl/mg wet wet weight/hr

Control Kidney		Perfused Kidney	
1.797	Glycerol Dextran Hanks sol.	1.696	94%
1.309		1.175	90%
2.192		2.694	123%
1.767	Dextran Hanks sol.	2.102	119%
1.795	DMSO Dextran Hanks sol.	2.225	124%
1.729		1.684	94%
1.508		1.148	76%
2.225		1.457	65%

The control values were obtained from the slices of the second unperfused kidney of the same cat.

was measured without freezing. The results were illustrated in Table 2. In the kidney perfused with Hanks solution which contains 4% dextran and 15 ml/dl glycerol, the oxygen consumption stayed in about normal range compared with each

Table 3. COMPARISON OF EFFECTS OF PERFUSATE ON TISSUE RESPIRATION

μl/mg wet weight/hr

Glycerol Dextran Hanks sol.		Dextran Hanks sol.
1.994	<	2.775
2.015	>	1.706
2.739	>	1.969
1.164	>	0.987
2.273	>	1.989
2.394	>	1.818
2.321	>	2.052
3.039	<	3.846

One kidney was perfused with 4% dextran Hanks solution. The other kidney of the same cat was perfused with 4% dextran Hanks solution added 15 ml/dl glycerol.

control value, although individual tissue slices showed infrequently some wide variation either to dextran or glycerol perfusate. Therefore, we assumed on the whole that remarkable change was not observed by even bloodless perfusion of dextran or glycerol. But by the perfusion with 15 ml/dl DMSO the tissue slices showed some decrease of oxygen uptake compared to the slices obtained from the kidney perfused with dextran or glycerol.

In some cases one kidney of a cat was perfused with 4% dextran Hanks solution and another with 15 ml/dl glycerol Hanks solution. The tissue slices of the kidney perfused with glycerol showed higher rate of respiration than those with dextran as illustrated in Table 3.

Comparing the effect of dextran and glycerol on the histological examination, it was observed an expansion of the interstitial tissue in both many cases (Fig. 1). Unidentified substance stained with eosin was found in Bowman's capsule (Fig. 2) in about half of all the kidneys perfused with glycerol, but in those with dextran, it was found only in a few cases.

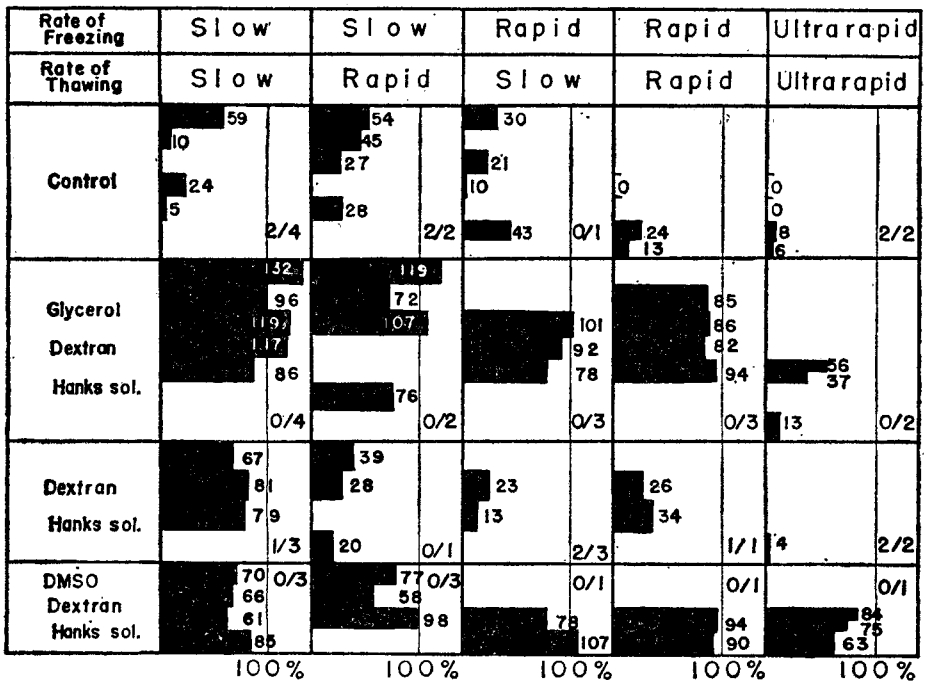
Relation between freezing and thawing rate and the cryoprotective effect of additives.

The rate of freezing and thawing was established in three grades as described above. The cryoprotective effects of 4% dextran, 15 ml/dl glycerol and 15 ml/dl DMSO were compared under five conditions, i. e. s-f : s-t, s-f : r-t, r-f : s-t, r-f : r-t, u-f : u-t as illustrated in Table 4, and showed the following results.

First, the kidney perfused with the solution containing additives showed higher oxygen uptake than the control kidney. Second, in spite of the facts that these cryoprotective agents showed the best protective action under the u-f : u-t in the case of single cells such as red blood cells or isolated cells as well known, in the use of tissue, however, it was proved that the tissue showed the least damage at slow rate of freezing and thawing (2°C/min). Third, compared the cryoprotective effect of these three additives, 4% dextran showed the least protective action and it was effective only in the condition of s-f : s-t. Fourth, generally slices treated with 15 ml/dl DMSO showed relative high oxygen uptake for every condition. Fifth, addition of 15 ml/dl glycerol showed marked protective action at s-f : s-t, though at u-f : u-t it was less effective than DMSO.

On histological examination, most severe injury caused by freezing and thawing was the formation of large vacuoles which appeared in tubular cells. The damage was manifested in the cases of freezing and thawing in which the oxygen uptake showed low value, such as contral and dextran at u-f : u-t (Fig. 4). On the other hand, those large vacuoles were hardly seen in all the cases of tissues perfused with DMSO and glycerol (Fig. 5). The denominator of the fraction illustrated in each column on Table 4 shows the number of vacuole formation. With regard to the slice obtained from the kidney perfused with glycerol and DMSO, histological examination agreed with the results of tissue respiration, but in the cases of perfusion with dextran and control the both results did not correspond exactly. With the exception of u-f : u-t, it was difficult to find any histological difference between four different freezing and thawing conditions in which the kidney was perfused with glycerol.

Table 4. COMPARISON OF THE CRYOPROTECTIVE EFFECTS OF 4% DEXTRAN, 15 ml/dl GLYCEROL AND 15 ml/dl DMSO UNDER FIVE CONDITIONS



Bars and integers illustrated in each column indicate per cent of the rate of tissue oxygen uptake against non-freezing control. The denominator of fractions illustrated in each column shows the number of cases examined histologically and the numerator shows the cases of vacuole formation.

DISCUSSION

In order to evaluate the results of freezing experiment of the kidney many investigators have observed the kidney function induced by autotransplantation of the frozen kidney and also referred to histological examination of the tissue. The author intended to examine the effect of cryoprotective agents which were given by vascular route. To estimate the extent of the tissue survival, the tissue respiration and histological examination were employed.

I will start to discuss the circumstances which would affect the evaluation of the results. The first, the time elapsed between extirpation of the kidney and the start of freezing, measurement of oxygen uptake or fixation, and between thawing and measurement of oxygen uptake or fixation, is an important factor and hardly neglected. For example, when the kidney was stored at 4°C, the rate of tissue respiration was not so much affected for the first 10 hrs, but histological changes

which was most remarkable in tubular cells appeared in a short time. This time factor is an important requisite for evaluation of the results of histological examination on the living material fixed after freezing and thawing. Optical microscope was not so reliable to detect delicate changes which might be produced by freezing and thawing, that gross changes such as vacuole formation were preferred in proof of deterioration in this experiment. Jumping at a conclusion, it would not be enough to measure oxygen uptake and to examine histological changes, for deciding whether cells are still alive or not, but transplantation or culture of the frozen tissue would be a conclusive factor.

The next point to be considered is the effect of the simple perfusion with artificial fluid[6)10)11)] on the tissue, which is the fundamental procedure of the author for isolation of an organ from the body. Huggins[7)] reported on the tissue injury by perfution with glycerol, but Barner[2)] could not confirm the observation. In our experiment, it was hard to say that the tissue did not get any effect by perfusion. For instance, the value of the tissue respiration were scattered by simple perfusion with cold artificial fluid. It was also the extreme case with perfusion of DMSO, it took the value between 120% and 65% of each control, and on histological examination the change was found in the Bowman's capsule.

Furthermore, following factors may be attributed to the effective causes of fluctuation in experimental results. The first, it may concern with the modes of perfusion. Our experiments showed that perfusion with pulsatory pressure was more effective than with constant pressure for the purpose of replacing blood by artificial fluid and the tissue damage was more severe when the pulsation pressure is higher than the normal blood pressure. By this reason, the author always regulates the pulsation pressure at 90/70 mmHg. As Huggins clearly showed osmotic pressure change by adding glycerol or DMSO to the perfusate causes injuries by swelling in kidney, even if the pulsation pressure is strictly controlled. In our experiment, however, the used basic perfusate has been given enough consideration on this point by adding dextran in Hanks solution for not causing tissue swelling nor shrinkage *per se*, but rather producing tissue shrinkage after perfusion of glycerol or DMSO solution. The second, the temperature of perfusate is a variable which is desirable to be fixed. It is preferable to be below 10°C[21)] during perfusion; in this experiment attention was paid to bring the perfusate temperature at 5°C. The third, the structural characteristics of blood vessels in the kidney is also an unrestrained condition. The time required from the start of perfusion to the disappearance of red blood cell in the venous flow depends on the extent of the shunt flow or the rotation of flowing, sometimes it is hard to expect how long it should be perfused. Also it was noticed that the time required to replace blood by artificial fluid depended on the small surgical treatment, whether the branch vessels around kidney had been ligated and cut before or after the start of perfusion. Such delicacy of circulation means difficulty of distribution of cryoprotective agent evenly into every corner of the kidney. With the local difference of distribution which might have happened, it would reasonably affect on the rate of respiration or histological findings after freezing and thawing. For all the above stated reasons, it gives some trouble to make a decided conclusion.

It was reported that dextran showed very good cryoprotective action in preservation of kidney at low temperature by Block et al[4]. But the author observed that dextran was effective only under the condition of slow freezing and slow thawing. The results in this experiment agreed well with that of experiment with bone marrow by Rowe[15], in that dextran, glycerol and DMSO showed a marked cryoprotective action at slow freezing and thawing. In our case, however, the suspended temperature at ultrarapid freezing lowered until −79°C, and at slow freezing it was −8°C or −12°C. This difference in the lowest temperature may be one of the factors why tissue showed the least damage at slow rate of freezing and thawing.

As mentioned above, it would be required to establish well-defined experimental conditions of freezing and thawing for each living system. Accordingly, it cannot be applied the results obtained with cells to tissues. It is also a natural consequence that the effects of a cryoprotective agent are not the same by the route, through which it is administered, either diffuse into tissue from the surface or given through vessel. In the latter case, it is the most important requisite that vascular system is protected from injuries which may be produced by additives. Furthermore, it would be an inevitable undesirable factor that there is difference of the rate of heat conduction in place to place of the whole organ because of its mass. By treating those problems as possible as quantitatively, the author has tried to find out the clue, for what should be the best way to estimate the extent of the tissue or organ survival after frozen and thawed.

SUMMARY

1. The kidney of a cat was perfused *in situ* with 4% dextran Hanks solutions and it was sliced and frozen.

2. Tissue respiration and histological examination were adopted for evaluation of the tissue survival after freezing and thawing.

3. Cryoprotective effects of 4% dextran (mol. wt. 63,000), 15 ml/dl glycerol and 15 ml/dl DMSO were examined by administration through the vascular system.

4. The rate of freezing was established at three grades; as slow freezing 2°C lowering per minute, as rapid freezing 25°C per minute and as ultrarapid freezing 800°C per minute were performed. The rate of thawing was also controlled with three grades; as slow thawing 2°C raising per minute, as rapid thawing 45–55°C per minute and as ultrarapid thawing 1000°C per minute were employed. Five conditions were established with combination of the rate of freezing and thawing.

5. Some tissue damage was observed in the kidney which was perfused with the artificial fluid but not frozen.

6. Generally, tissue seemed to show the least damage at slow rate of freezing and thawing.

7. It would be concluded as follows by comparison of effectiveness of the three different kinds of cryoprotective agents. Dextran showed the least protective action, i. e. it showed a considerable effect under slow rate of freezing and thawing. With regard to DMSO relative high protective effect was observed on an average for every condition, Glycerol showed, in general, marked protective effect, especially

at slow freezing and slow thawing, though it was a little less effective than DMSO at ultrarapid freezing and thawing.

8. For the expectation of tissue survival it was concluded that histological examination was more useful than measurement of tissue respiration.

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REFERENCES

1. ASAHINA E.
Fed. Proc. 1965. 24s. 183/187
Freezing process and injury in isolated animal cells.
2. BARNER H. B., RIVERS R. J., CADY B. and WATKINS E.
Surgery. 1963. 53. 344/347
Survival of the canine ureter after freezing.
3. BELTRAN J. C. and BLUMENTAL H. T.
J. Urol. 1959. 82. 424/435
Effects of dehydration-freezing-thawing on the preservation and homotransplantability of renal tissue.
4. BLOCH J. H., LONGERBEAM J. K., MANAX W. G., HILAL S. and LILLEHEI R. C.
Trans. Amer. Soc. artif. intern. Organs. 1963. 9. 139/147
Preservative solutions for freezing whole organs in vitro.
5. CHAPLIN J. H. and MOLLISON P. L.
Lancet. 1953. 26. 215/218
Improved storage of red cells at −20°C.
6. HALASZ N. A., SEIFERT L. N. and ORLOFF M. J.
Surgery. 1966. 60. 368/372
Whole organ preservation.
1. Organ perfusion studies.
7. HUGGINS C. E.
J. Physiol. 1959. 145. 54
The effects of varying concentrations of glycerol on the weight of isolated rabbit kidney slices.
8. LOVELOCK J. E.
Biochem. biophs Acta. 1953. 11. 28/36
The mechanism of the preotective action of glycerol against haemolysis by freezing and thawing.
9. LOVELOCK J. E. and BISHOP M. W. H.
Nature. 1959. 183. 1394/1395
Preservation of freezing damage to living cells by dimethyl sulfoxide.
10. MANAX W. G., BLOCH J. H., LONGERBEAM J. K. et al
Surgery. 1964. 56. 275/282
Successful 24 hours in vitro preservation of canine kidneys by the combined use of hyperbaric oxygenation and hypothermia.

11. MANAX W. G., BLOCH J. H., LARGIADER F., LYONS G. W., EYAL A., LILLEHEI R. C.
Surgery. 1965. 57. 528/534
Organ perfusion before transplantation with particular reference to the kidney.
12. MAZUR P.
Fed. Proc. 1965. 24s. 175/182
Causes of injury in frozen and thawed cells.
13. MERYMAN H. T.
Science. 1965. 124. 515/521
Mechanics of freezing in living cells and tissues.
14. MERYMAN H. T.
Cryobiology. 1966. 2. 165/170
The interpretation of freezing rates in biological materials.
15. ROWE A. W.
Cryobilogy. 1966. 3. 12/18
Biochemical aspects of cryoprotective agents in freezing and thawing.
16. SMITH A. U.
Exp. Cell Res. 1953. 3. 574/583
Cultivation of ovarian granulosa cells after cooling to very low temperatures.
17. SMITH A. U. and PARKS A. S.
Lancet. 1951. 570/572
Preservation of ovarian tissue of low temperatures.
18. SMITH A. U.
London Edward Arnord. 1961
Biological Effects of Freezing and Supercooling.
19. SMITH A. U.
Fed. Proc. 1965. 24s. 196/203
Problems in freezing organs and their component cells and tissues.
20. SUDA I., ADACHI C. and KITO K.
Kobe J. Med. Sci. 1963. 9. 41/67
Studies on the isolated cat's brain in vitro.
1. Isolation methods and observation on the spontaneous electrical activity.
21. SUDA I., KITO K. and ADACHI C.
Nature. 1966. 212. 268/270
Viability of long term frozen cat brain in vitro.

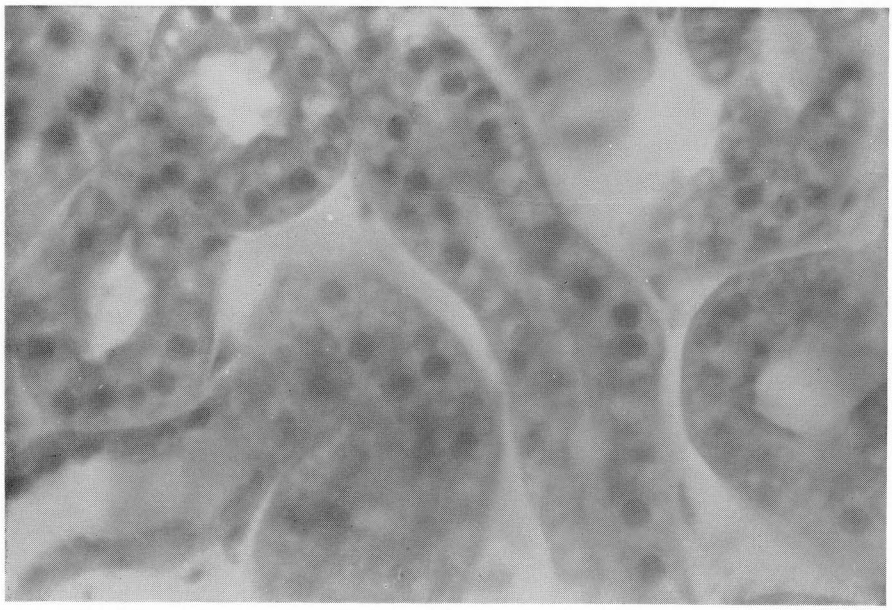

Fig. 1 Microphotograph of the tubulus of the kidney perfused with 4% dextran Hanks solution added 15 ml/dl glycerol. Enlargement of the interstitial tissue is seen.

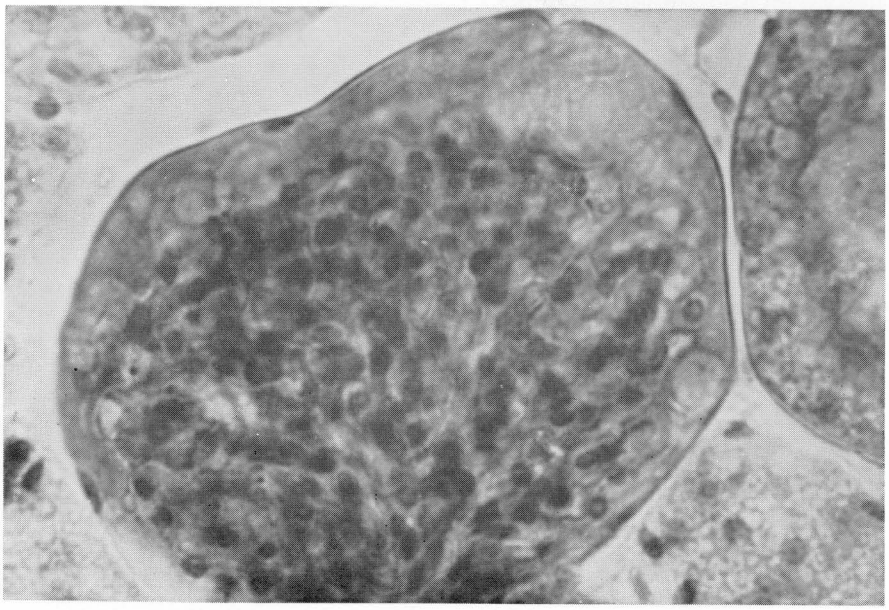

Fig. 2 Microphotographs of the glomerulus. Unidentified substance stained with eosin is seen in the Bowman's capsule.

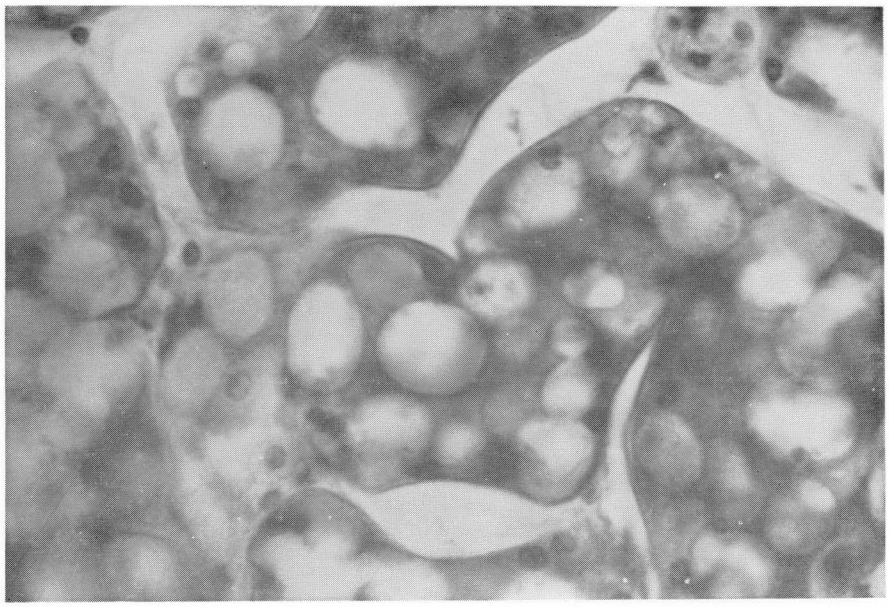

Fig. 3 Microphotographs of the tubulus of the control kidney frozen and thawed at slow freezing-rapid thawing rate. Formation of vacuole is seen.

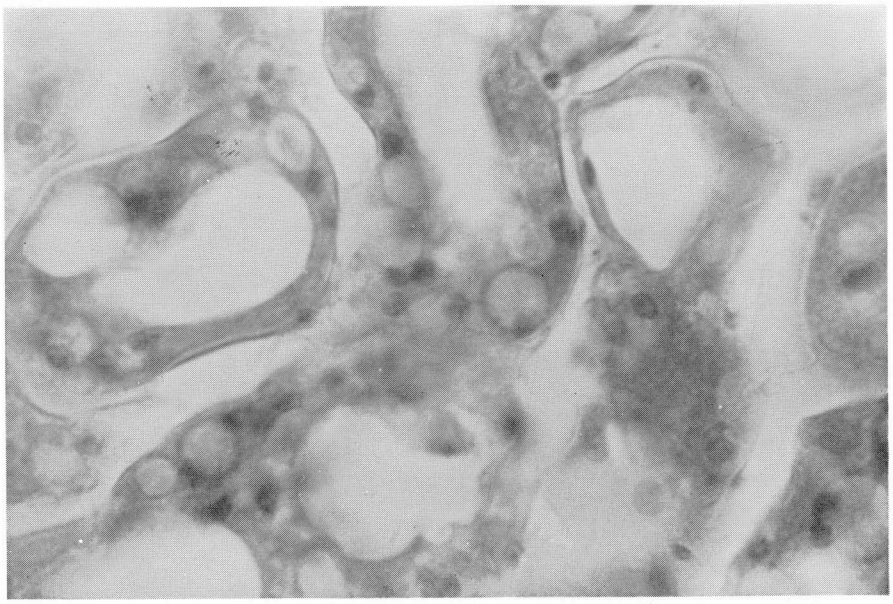

Fig. 4 Microphotographs of the tubulus of the control unperfused kidney frozen and thawed at ultrarapid freezing-ultrarapid thawing rate. Formation of large vacuole is seen.

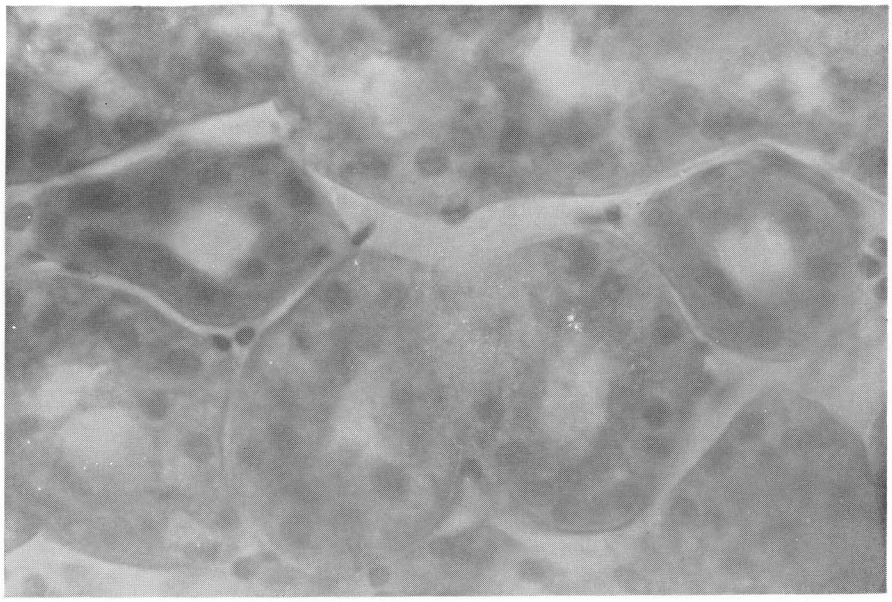

Fig. 5 Microphotographs of the tubulus of the kidney perfused with 4% dextran Hanks solution added 15 ml/dl glycerol and frozen and thawed at rapid freezing–rapid thawing rate. Formation of vacuole is not seen.