

# Damage to Pork Liver Caused by Repeated Freeze-Thaw Cycling and Refrigerated Storage

ELIZABETH D. STRANGE, SUSAN B. JONES, and ROBERT C. BENEDICT

## ABSTRACT

The effects of freezing and thawing (F-T) and refrigerated storage (R-S) on pork liver were examined. Intact cells were isolated from the tissue and analyzed for membrane integrity and the tissue extracts were analyzed for protein content. Significant ( $p < 0.05$ ) differences were noted in the number of isolated intact cells and in tissue protein content between F-T and R-S or fresh livers. Differences in cell membrane integrity were also noted between fresh and R-S livers. Repeated freezing and thawing appears to break down the liver structure differently than does refrigerated storage.

## INTRODUCTION

IN 1978 the United States exported approximately \$205 million worth of variety meats but experienced rejection rates of 5-30% at port of entry as "unfit for human consumption" or for other reasons (Turczyn, 1980). Miller and Bongers (1981) stated that blood leakage or stains on boxes indicated that the product had been defrosted after initial freezing. Livers were the variety meat most often rejected (Private Communication, T. H. Camp).

Although important economically, the deterioration of liver during storage, freezing, and thawing has not been examined extensively. Smith et al. (1983a) reported that freezing temperature had little effect on weight loss, color score, overall appearance, odor, and tenderness of beef livers. Smith et al. (1983b) also indicated that vacuum packaging as soon as possible after slaughter gave the most acceptable appearance to chilled beef liver destined for retail display.

Two recent studies (Berry et al., 1982; Hanna et al., 1982) showed that frozen storage had no significant effect on the total microbial populations (aerobic plate counts) of stored variety meats. The study by Berry et al. (1982) included transportation at above freezing temperatures following freezing. Hamm and Masic (1975) reported on a method to distinguish fresh from frozen liver, and Partmann (1973) examined the histological effects of different freezing and thawing rates. Heinz (1974) contrasted frozen-stored liver with fresh liver, and reported that freezing had no adverse effects on the use of liver in products, but that freezing and thawing increased the amount of drip. Smith et al. (1983a) found that uncut frozen beef livers had less drip than sliced frozen liver. Smith et al. (1983b), however, showed that frozen and thawed liver had less weight loss than did chilled liver.

The objective of this study was to measure the damage to liver tissue and to elucidate patterns of degradation during refrigerated storage and after repeated freezing and thawing.

## MATERIALS & METHODS

WHOLE PORK LIVERS were obtained from a local slaughterhouse immediately following Federal inspection and removal of the bile

duct and gall bladder. Each liver was divided into seven samples (approximately 125-150g). The samples were vacuum packaged and sealed with a Smith SuperVac in 7 X 7 sized "IKD ALL-VAC 13 FBR" pouches. Temperature was monitored by inserting a YSI temperature probe into the center of a tissue sample and continuously recording temperatures. Samples were stored at either  $-20^{\circ}\text{C}$  or  $+5^{\circ}\text{C}$  within 3 hr of slaughter. The sample from each liver which was analyzed fresh was not vacuum packaged. A total of six whole pork livers were used: three for freeze-thaw cycling (F-T) and three for refrigerated storage (R-S).

Freeze-thaw cycled liver samples were initially frozen for 70 hr at  $-20^{\circ}\text{C}$  in a freezer-incubator. The freezer-incubator was then programmed to hold the temperature at  $+5^{\circ}\text{C}$  for 24 hr, followed by  $-20^{\circ}\text{C}$  for 24 hr. Each 48 hr freezing-refrigerated storage period was designated as one freeze-thaw cycle. This cycling was continued for 11 days for a total of six cycles. Liver samples were analyzed at the end of the thaw portion of each cycle.

The refrigerated storage liver samples were placed in the freezer-incubator set at  $+5^{\circ}\text{C}$ . Refrigerated storage of 24 hr was called a cycle. Frozen-thawed and refrigerated storage samples with the same cycle number have equivalent time in refrigerated storage.

Livers were monitored to assess drip formation, number of isolated cells and their viability, and protein content of the tissue extracts. Analysis was carried out on day of slaughter (fresh) (except for drip) and after 1, 2, 4, 5, and 6 F-T or R-S cycles.

The amount of drip formed at each cycle was determined by weighing the drained liver sample and measuring the amount of liquid left in the package. Percentage drip was reported as (volume liquid (mL)/weight drained sample (g) + weight of liquid (g)) X 100.

Phosphate buffered saline (PBS) contained 0.2g KCl, 0.2g  $\text{KH}_2\text{PO}_4$ , 2.89g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and 7.0g NaCl per liter (pH 7.4). PBS + 0.5 mM EGTA (ethyleneglycol-bis(-amino-ethyl ether) N,N'-tetracetic acid) and 1% w/v Triton X100 in PBS were also prepared.

Modified magnesium-free Hanks balanced salt solution (HBSS) contained 0.4g KCl, 0.06g  $\text{KH}_2\text{PO}_4$ , 0.12g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 8.0g NaCl, 2.19g  $\text{NaHCO}_3$  per liter (pH 7.4) (Moldeus et al., 1978). The 0.05% collagenase Type IV (Sigma) - 0.01% hyaluronidase Type II (Sigma) solution was prepared immediately before use in HBSS + 5 mM  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ .

A filtered 0.5% trypan blue solution in PBS was diluted with an equal volume of PBS immediately before use. A 200- $\mu\text{L}$  aliquot of a 0.5% solution of fluorescein diacetate (FDA) in acetone was diluted with PBS to a final concentration of 0.5  $\mu\text{g}/\text{mL}$  (Rotman and Papermaster, 1966).

Cell isolations were carried out in duplicate. The liver cell isolation procedure (Fig. 1) was adapted from that of Fry et al. (1976). Three grams of 0.5-mm thick liver slices (about 20 slices) were prepared with a Stadie-Riggs tissue slicer. As each slice was cut, it was placed into a tared 125-mL Erlenmeyer flask containing 10 mL PBS. Slicing took about 15 min per isolation. The sliced liver tissue was washed two times with 10 mL PBS for 10 min each time. The sample flasks were placed in a  $37^{\circ}\text{C}$  water bath and shaken at 100 RPM for tissue washings and enzymatic digestion. The PBS was decanted and labeled PBS. Washing was continued with two 10-mL portions PBS + 0.5 mM EGTA, a  $\text{Ca}^{+2}$  chelating buffer, for 10 min each, and this decantate was labeled PBS-EGTA. The washed liver slices were then digested for 60 min with 10 mL enzyme solution. After completion of the incubation, the cells were released from the liver tissue by gentle agitation with a stirring rod on a 100-mesh screen in a Collector. The liver tissue in the Collector was washed twice with 5-mL portions ice cold PBS. Tissue debris remaining on the screen was discarded. The filtrate contained the liver cells and cell debris. Cells were separated from the cell debris by centrifugation at 80 X g for 5 min and the pellets washed three times in 10-mL portions cold PBS for a total centrifugation time of 20 min. The supernate from

the pellets was saved and labeled CELL DEBRIS. The isolated cells were then suspended in PBS, and kept cold until counting and protein determinations were completed.

A Hausser Hy-Lite Ultra Plane hemocytometer was used for cell counting. Isolated cells were treated with FDA by adding 100  $\mu$ L of an appropriate dilution of the cells to 9.9 mL FDA solution. The fluorescing cells were counted immediately using blue excitation illumination at 125X magnification. Fluorescing cells are assumed to have an intact cell membrane (Rotman and Papermaster, 1966). Cells visible by phase contrast microscopy also were counted in the same hemocytometer chamber at the same magnification.

Isolated cells were stained with trypan blue by adding 100  $\mu$ L of an appropriate dilution of the cell suspension to 9.9 mL trypan solution. After staining for 1 min, cells were counted under bright field illumination and then under phase contrast. Viable cells with an intact cell membrane exclude trypan (Patterson, 1979). Phase counts were reported as number of cells  $\times 10^7$ /3g liver tissue and the ratios of FDA count-to-phase count and of trypan count-to-phase count were calculated and reported. All round particles visible in the microscope field were counted.

Biuret protein determinations were performed on all extracts and isolated cell fractions. Isolated cell fractions were solubilized with 1% Triton X100 before biuret determination. A biuret protein standard (bovine serum albumin) was run daily.

For histologic examination, tissue samples were fixed in pH 7, 0.08M PO<sub>4</sub> buffered, 10% formalin before sectioning with a freezing microtome. The 20 micron sections were stained with hematoxylin and eosin, mounted, and photographed at 50X with a Nikon Opti-photo microscope.

Differences in means of parameters measured were evaluated by

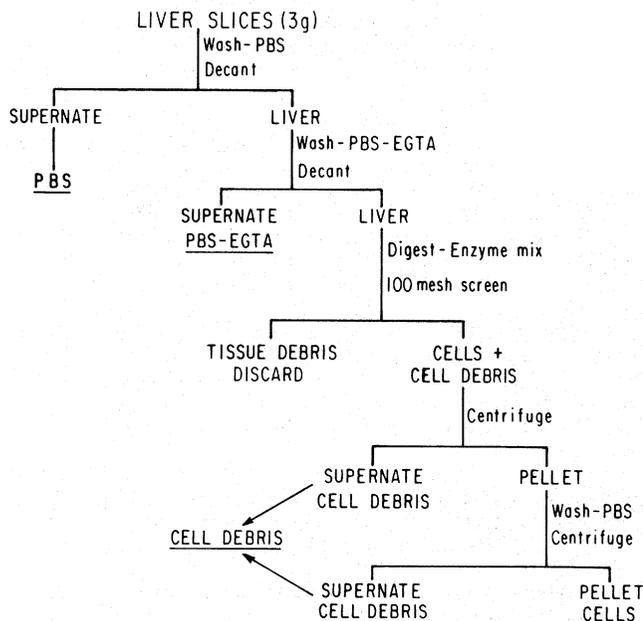


Fig. 1—Flow diagram for cell isolation procedure. Fractions underlined were measured for protein content.

the “t” test described in Steel and Torrie (1980). Linear cycling effects were determined by analysis of variance using a general linear models procedure. Significance was tested at the  $p < 0.05$  level.

## RESULTS & DISCUSSION

THE TEMPERATURE VS TIME curve for typical 48-hr freeze-thaw cycle is shown (Fig. 2). The time for sample freezing or thawing varied with sample size, and the slope of the line generated by monitoring the temperature of liver samples during freezing and/or thawing was not constant. The temperature/time curve flattened when the sample reached  $-2^{\circ}\text{C}$  because of the phase change of liquid H<sub>2</sub>O to ice. The time at  $-2^{\circ}\text{C}$  was extended during the thawing portion because of a smaller temperature differential between sample and ambient temperatures. Hamm et al. (1982) reported that super-cooling effects were possible during the freezing of meat products and caused an increase in the overall rate of freezing. We found no evidence for this effect in the monitoring of our samples (Fig. 2).

Percent drip increased for both F-T and R-S livers and had a significant linear relationship with cycling (Table 1). While freeze-thaw cycling produced more drip than refrigerated storage, these differences were not significant. The differences between fresh and F-T liver, however, were significant at cycles 4, 5, and 6, and the differences between fresh liver and R-S liver were significant at cycle 1 (Table 1). Smith et al. (1983b) showed that frozen-thawed whole beef livers had less weight loss than did chilled livers. Holding time for chilled livers was 15.5 days, and the frozen-thawed livers were frozen only once. Weight loss from drip increased as a function of holding time and drip from F-T cycle 1 was less than drip from R-S cycle 6 (3.1% vs 5.7%).

The cell isolation procedure (Fig. 1) involved stepwise detachment of the cells from each other and from the collagen matrix which surrounds the cells. The PBS wash removed protein not tightly held within the liver structure. The PBS-EGTA wash disrupted the desmosomes, part of

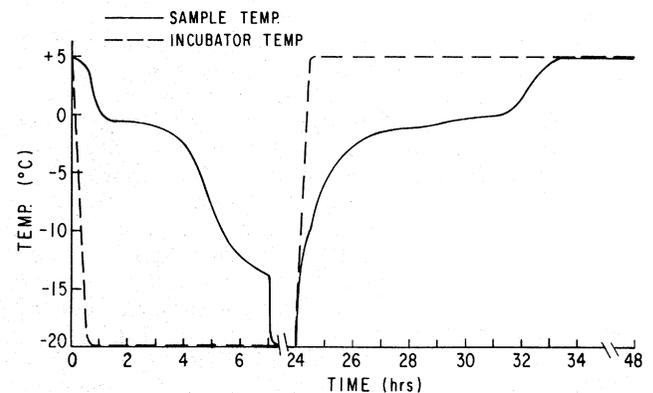


Fig. 2—Temperatures during a typical 48-hr freeze-thaw cycle.

Table 1—Mean percent drip and standard deviations for freeze-thaw (F-T) (N = 3) and refrigerator stored (R-S) (N = 3) livers

|                  | Fresh<br>% (S.D) | Cycle 1<br>% (S.D.)                          | Cycle 2<br>% (S.D.) | Cycle 4<br>% (S.D.)    | Cycle 5<br>% (S.D.)     | Cycle 6<br>% (S.D.)     |
|------------------|------------------|--|---------------------|------------------------|-------------------------|-------------------------|
| F-T <sup>a</sup> | 0 <sup>b</sup>   | 3.1 (2.1)                                    | 5.5 (3.6)           | 8.9 (2.4) <sup>c</sup> | 11.4 (5.3) <sup>*</sup> | 10.5 (4.8) <sup>*</sup> |
| R-S <sup>*</sup> | 0                | 1.9 (0.9) <sup>*d</sup><br>N.S. <sup>e</sup> | 2.7 (2.7)<br>N.S.   | 3.4 (3.7)<br>N.S.      | 5.9 (4.6)<br>N.S.       | 5.7 (5.6)<br>N.S.       |

<sup>a</sup>  $p < 0.05$  for the F value from analysis of variance for the linear effect of cycle on percent drip.

<sup>b</sup> Percent drip for fresh liver defined as 0.

<sup>c</sup> Probability of a larger value of  $t < 0.05$  for differences in percent drip between fresh and F-T livers.

<sup>d</sup> Probability of a larger value of  $t < 0.05$  for differences in percent drip between fresh and R-S liver.

<sup>e</sup> N.S. — not significant — probability of a larger value of  $t > 0.05$  for differences in drip between F-T and R-S livers.

the tissue structure involved in cell-to-cell adhesion (Seglen, 1979). The PBS-EGTA fraction contained protein not removed by the first PBS wash plus proteins which were solubilized or loosened by  $Ca^{+2}$  chelation. The enzymatic digestion loosened the collagen surrounding the cell and some intercellular junctions. Agitation on the Collector screen destroyed the gap and tight junctions between the cells (Van De Werve, 1980). The cell debris contained matter which did not sediment at 80 X g, and the cell fraction contained the isolable cells in 3g liver. Cell protein was the protein in the isolable cell fraction and total protein was the protein recovered in the tissue fractionation scheme.

Tables 2 and 3 present data obtained from the cell isolation procedure performed on fresh, freeze-thaw (F-T) and refrigerator stored (R-S) livers. F-T fresh livers were significantly different from R-S fresh livers only in the phase count-number of cells isolated (Tables 2 and 3). Livers used in the R-S study yielded significantly higher numbers of cells because they contained considerably more red blood cells (for unknown reasons) in the isolated cell fraction than the livers used in the F-T study. These red blood cells were included in the counts since they were identifiable only by size with the magnification used, and nonparenchymal liver cells of a similar size exist (Homma et al., 1982).

The relationship between cell count and cell protein content is shown in Fig. 3. The livers used for the F-T studies had a higher protein content per cell than did the livers used for the R-S studies, indicating that the R-S livers contained larger numbers of smaller cells.

The number of isolated cells (phase-count) showed a significant linear decrease as storage time or cycle number increased for both F-T and R-S livers. The differences

between the number of cells isolated from fresh liver and F-T liver, and from fresh liver and R-S liver were significant after 4, 5, and 6 cycles. The number of cells isolated from F-T livers were always significantly smaller than the number isolated from R-S livers (Table 2). Because the R-S group of livers had higher cell counts when fresh, the import of the differences between F-T and R-S is unclear.

To compare effects of the different storage regimes on the isolability of the liver cell, the relative yield of isolable cells was calculated as a ratio of number of cells obtained from stored livers to number of cells obtained from fresh livers (Table 2).

Relative yields of the isolable cells showed significant differences for all three types of comparisons. F-T livers had significantly smaller ratios than fresh liver by cycle 2, but the R-S livers showed no significant differences until cycle 5. The relative yield of isolable cells from F-T liver was always less than from R-S liver and was significantly different for cycles 4 and 5.

Cell protein showed a significant linear decrease as cycle number increased for both F-T and R-S livers (Table 3). Cell protein of F-T liver was significantly lower than fresh liver and R-S liver for all cycles. However, cell protein of R-S liver was significantly smaller than fresh liver at cycle 6 only (Table 3).

Differences in phase count, relative yield ratios, and cell protein indicated that freezing and thawing destroy the liver cell much more rapidly and to a greater extent than refrigerated storage. Each freezing and thawing cycle destroyed 20% of the remaining isolable liver cells so that after six freezing and thawing cycles only 25% of the liver cells remain intact. However, the increase in the amount of drip formed during freezing and thawing was much less

Table 2—Means and standard deviations of parameters measured on isolated cells for freeze-thaw (F-T) (N = 3) and refrigerator stored (R-S) (N = 3) livers

|  | Fresh             |         | Cycle 1      |                       | Cycle 2      |                       | Cycle 4      |                       | Cycle 5      |                       | Cycle 6      |                      |
|--|-------------------|---------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|----------------------|
| Phase count — number (#) of cells isolated x 10 <sup>7</sup> /3g of liver tissue |                   |         |              |                       |              |                       |              |                       |              |                       |              |                      |
|  | # (S.D.)          |         | # (S.D.)     |                       | # (S.D.)     |                       | # (S.D.)     |                       | # (S.D.)     |                       | # (S.D.)     |                      |
| F-T <sup>a</sup>   | 32.5              | (22.3)  | 25.2         | (16.5)                | 19.8         | (12.7)                | 9.3          | (2.5) <sup>b</sup>    | 6.4          | (1.4) <sup>*</sup>    | 7.8          | (4.0) <sup>*</sup>   |
| R-S <sup>*</sup>   | 129.2             | (27.4)  | 127.7        | (12.5)                | 104.2        | (12.6)                | 99.9         | (14.9) <sup>*c</sup>  | 81.1         | (25.0) <sup>*</sup>   | 62.8         | (13.6) <sup>*</sup>  |
|  | *d                |         | *            |                       | *            |                       | *            |                       | *            |                       | *            |                      |
| Relative yield of isolable cells <sup>e</sup>                                    |                   |         |              |                       |              |                       |              |                       |              |                       |              |                      |
|  | Ratio (S.D.)      |         | Ratio (S.D.) |                       | Ratio (S.D.) |                       | Ratio (S.D.) |                       | Ratio (S.D.) |                       | Ratio (S.D.) |                      |
| F-T <sup>f</sup>   | 1.00              | (0)     | 0.814        | (0.619)               | 0.623        | (0.214) <sup>*b</sup> | 0.394        | (0.204) <sup>*</sup>  | 0.295        | (0.190) <sup>*</sup>  | 0.267        | (0.064) <sup>*</sup> |
| R-S <sup>f</sup>   | 1.00              | (0)     | 1.031        | (0.276)               | 0.844        | (0.244)               | 0.793        | (0.145)               | 0.617        | (0.066) <sup>*c</sup> | 0.511        | (0.153) <sup>*</sup> |
|  | N.S. <sup>g</sup> |         | N.S.         |                       | N.S.         |                       | *d           |                       | *            |                       | N.S.         |                      |
| FDA/phase counts ratios <sup>h</sup>   |                   |         |              |                       |              |                       |              |                       |              |                       |              |                      |
| F-T  | 0.542             | (0.272) | 0.615        | (0.295)               | 0.576        | (0.147)               | 0.677        | (0.155)               | 0.716        | (0.145)               | 0.665        | (0.220)              |
| R-S  | 0.638             | (0.120) | 0.672        | (0.071)               | 0.612        | (0.073)               | 0.548        | (0.123)               | 0.492        | (0.111)               | 0.610        | (0.146)              |
|  | N.S. <sup>g</sup> |         | N.S.         |                       | *d           |                       | NS           |                       | *            |                       | N.S.         |                      |
| Trypan/phase counts ratios <sup>i</sup>  |                   |         |              |                       |              |                       |              |                       |              |                       |              |                      |
| F-T <sup>a</sup>   | 0.676             | (0.098) | 0.686        | (0.087)               | 0.732        | (0.089)               | 0.816        | (0.103) <sup>*b</sup> | 0.886        | (0.034) <sup>*</sup>  | 0.837        | (0.134) <sup>*</sup> |
| R-S <sup>a</sup>   | 0.592             | (0.085) | 0.766        | (0.067) <sup>*c</sup> | 0.771        | (0.048) <sup>*</sup>  | 0.729        | (0.045) <sup>*</sup>  | 0.741        | (0.016) <sup>*</sup>  | 0.738        | (0.049) <sup>*</sup> |
|  | N.S. <sup>g</sup> |         | N.S.         |                       | N.S.         |                       | NS           |                       | *d           |                       | N.S.         |                      |

a\* p < 0.05 for the F value from analysis of variance for the linear effect of cycle on parameter measured.

b\* Probability of a larger value of t < 0.05 for the differences in parameter measured between fresh and F-T livers.

c\* Probability of a larger value of t < 0.05 for the differences in parameter measured between fresh and R-S livers.

d\* Probability of a larger value of t < 0.05 for the differences in parameter measured between F-T and R-S livers.

e Ratio of the number of cells isolated at the specified cycle to the number of cells isolated when the same liver was fresh.

f Analysis of variance for linear effect not done.

g N.S. — not significant — probability of a larger value of t > 0.05 for the differences in parameter measured between F-T and R-S livers.

h Ratio of the number of cells visible when viewed with fluorescent illumination and stained with FDA to the number of cells visible under phase contrast illumination.

i Ratio of the number of cells visible when viewed under bright field illumination and stained with Trypan Blue to the number of cells visible under phase contrast illumination.

## CONCLUSIONS

FREEZING AND THAWING liver tissue caused extensive damage to the liver cells but preserved some of the ability of surviving cells to exclude trypan blue during the first two cycles. Each freeze-thaw cycle destroyed about 20% of the remaining intact cells, but did not destroy the supporting collagen matrix. Damage to the cell-cell adhesion mechanisms was evident after one freeze-thaw cycle.

Damage to the liver tissue also occurred during refrigerated storage, but it was only after 4 days of storage that the rate of cell destruction matched that of F-T; damage to the cell-cell adhesion mechanisms was significant. Autolytic damage to the cells, as measured by viability, is a significant factor during refrigerated storage of liver.

## REFERENCES

- Berry, B.W., Rothenberg, C.A., Oblinger, J.L., Miller, W.R., Bongers, A.J. and Labots, H. 1982. Changes in the microbiological and shelf-life characteristics of beef tongues and livers following transcontinental and transoceanic shipment. *Meat Sci.* 7: 81.
- Camp, T.H. 1983. Private communication. USDA-ARS, Meat Processing and Marketing Research, College Station, TX.
- Fry, J.R., Jones, C.A., Wiebkin, P., Bellemann, P., and Bridges, J.W. 1976. The enzymic isolation of adult rat hepatocytes in a functional and viable state. *Anal. Biochem.* 71: 341.
- Hamm, R., Gottesmann, P., and Kijowski, J. 1982. Einfrieren und Auftauen von Fleisch: Einflüsse auf Muskelgewebe und Tausaftbildung. *Die Fleischwirtschaft* 62(8): 983.
- Hamm, R. and Masic, D. 1975. Routinemethode zur Unterscheidung zwischen frischer Leber und aufgetauter Gefrierleber. *Die Fleischwirtschaft* 55(2): 242.
- Hanna, M.O., Smith, G.C., Savell, J.W., McKeith, F.K., and Vanderzant, C. 1982. Microbial flora of livers, kidneys and hearts from beef, pork and lamb: effects of refrigeration, freezing and thawing. *J. Food Prot.* 45(1): 63.
- Heinz, G. 1974. Qualitätserhaltung von Rinderleber unter Kühl- oder Gefrierbedingungen. *Die Fleischwirtschaft* 54(4): 670.
- Homma, S., Nagamori, S., Hasumura, S., Fujise, K., Sujino, H., Kameda, H., Kirino, Y., Hataba, Y., and Suzuki, T. 1982. The morphological studies on characteristics of isolated parenchymal and non-parenchymal cells of the rat liver. *J. Electron Microsc.* 31: 389.
- Miller, W.R. and Bongers, A.J. 1981. Receivers' packaging preferences and packaging deterioration problems of U.S. variety meats observed at European markets. USDA, *Advances in Agricultural Technology* 6.
- Moldeus, P., Hogberg, J. and Orrenius, S. 1978. Isolation and use of liver cells. *Methods in Enzymology* 52: 60.
- Partmann, W. 1973. Histologische Veränderungen in Rind- und Schweinefleisch sowie Schweineleber unter definierten Gefrier- und Auftaubedingungen. *Die Fleischwirtschaft* 53(1): 65.
- Patterson, M.K. 1979. Measurement of growth and viability of cells in culture. *Methods in Enzymology* 58: 141.
- Rotman, B. and Papermaster, B.W. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. N.A.S.-Biochem.* 55: 134.
- Seglen, P.O. 1979. Hepatocyte suspensions and cultures as tools in experimental carcinogenesis. *J. Toxicol. Environ. Health* 5: 551.
- Smith, G.C., Purser, D.E., Savell, J.W., Stiffler, D.M., and Dutson, T.R. 1983a. Effect of freezing temperature on weight loss and quality characteristics of beef liver, kidney, heart, and tongue. *J. Food Sci.* 48: 1726.
- Smith, G.C., Savell, J.W., Stiffler, D.M., Johnson, D.D., and Vanderzant, C. 1983b. Weight loss and quality characteristics of frozen versus chilled beef livers and hearts. *J. Food Sci.* 48: 1728.
- Steel, R.G.D. and Torrie, J.H. 1980. "Principles and Procedures of Statistics." McGraw-Hill Book Company, Inc., New York.
- Turczyn, M.T. 1980. Guidelines for packaging frozen edible offal for export. U.S. Department of Agriculture. Marketing Research Report Number 1115.
- Van De Werve, G. 1980. Isolation and characteristics of hepatocytes. *Toxicology* 18: 179.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Table 3—Means and standard deviations of protein content of isolated cells and of tissue extracts for freeze-thaw (F-T) (N = 3) and refrigerator stored (R-S) (N = 3) livers

|                                  | Fresh<br>mg (S.D.) | Cycle 1<br>mg (S.D.)   | Cycle 2<br>mg (S.D.) | Cycle 4<br>mg (S.D.)   | Cycle 5<br>mg (S.D.) | Cycle 6<br>mg (S.D.)   |
|----------------------------------|--------------------|------------------------|----------------------|------------------------|----------------------|------------------------|
| Cell protein <sup>a</sup>        |                    |                        |                      |                        |                      |                        |
| F-T* <sup>b</sup>                | 177 (51)           | 97 (16)* <sup>c</sup>  | 75 (45)*             | 37 (8)*                | 34 (3)*              | 38 (14)*               |
| R-S* <sup>b</sup>                | 246 (93)           | 266 (72)               | 260 (69)             | 193 (20)               | 207 (97)             | 157 (19)* <sup>d</sup> |
|                                  | N.S. <sup>e</sup>  | * <sup>f</sup>         | *                    | *                      | *                    | *                      |
| PBS protein <sup>a</sup>         |                    |                        |                      |                        |                      |                        |
| F-T                              | 228 (8)            | 357 (75)* <sup>c</sup> | 309 (46)*            | 275 (37)*              | 271 (37)*            | 233 (40)               |
| R-S* <sup>b</sup>                | 233 (28)           | 240 (25)               | 245 (24)             | 288 (65)* <sup>d</sup> | 286 (17)*            | 358 (50)*              |
|                                  | N.S. <sup>e</sup>  | * <sup>f</sup>         | *                    | N.S.                   | N.S.                 | *                      |
| PBS-EGTA protein <sup>a</sup>    |                    |                        |                      |                        |                      |                        |
| F-T* <sup>b</sup>                | 50 (2)             | 89 (4)* <sup>c</sup>   | 93 (10)*             | 82 (6)*                | 99 (9)*              | 110 (2)*               |
| R-S* <sup>b</sup>                | 44 (7)             | 60 (10)                | 45 (8)               | 79 (20)* <sup>d</sup>  | 72 (5)*              | 75 (9)*                |
|                                  | N.S. <sup>e</sup>  | * <sup>f</sup>         | *                    | N.S.                   | *                    | *                      |
| Cell debris protein <sup>a</sup> |                    |                        |                      |                        |                      |                        |
| F-T* <sup>b</sup>                | 284 (44)           | 280 (10)               | 258 (54)             | 226 (34)* <sup>c</sup> | 214 (4)*             | 205 (15)*              |
| R-S                              | 221 (76)           | 216 (47)               | 187 (149)            | 181 (68)               | 172 (65)             | 190 (84)               |
|                                  | N.S. <sup>e</sup>  | N.S.                   | N.S.                 | N.S.                   | N.S.                 | N.S.                   |
| Total protein <sup>g</sup>       |                    |                        |                      |                        |                      |                        |
| F-T <sup>h</sup>                 | 713 (33)           | 809 (31)* <sup>c</sup> | 722 (37)             | 606 (22)*              | 604 (18)*            | 572 (19)*              |
| R-S <sup>h</sup>                 | 730 (55)           | 767 (40)               | 724 (74)             | 726 (42)               | 723 (55)             | 766 (42)               |
|                                  | N.S. <sup>e</sup>  | N.S.                   | N.S.                 | * <sup>f</sup>         | *                    | *                      |

a Protein measured by biuret reaction.

b\*  $p < 0.05$  for the F value from analysis of variance for the linear effect of cycle on parameter measured.

c\* Probability of a larger value of  $t < 0.05$  for the differences in parameter measured between fresh and F-T livers.

d\* Probability of a larger value of  $t < 0.05$  for the differences in parameter measured between fresh and R-S livers.

e N.S. — not significant — probability of a larger value of  $t > 0.05$  for the differences in parameter measured between F-T and R-S livers.

f\* Probability of a larger value of  $t < 0.05$  for the differences in parameter measured between F-T and R-S livers.

g Calculated by summing the protein measurements made on the individual fractions that were isolated. Standard deviation determined by taking the square root of the sum of the variances and dividing by the number of fractions.

h Analysis of variance for linear effect not done.

than would be expected if drip contained most of the exudate from the destroyed cells. Both storage regimes eventually destroyed the liver cell to the extent that it could no longer be isolated by enzymatic techniques.

Viability of the isolated liver cells was estimated by two methods: FDA/phase count ratio and trypan/phase count ratio. Both methods indicated that approximately 50% of the isolated cells from fresh liver were viable.

This viability was lower than that reported by Fry et al. (1976) because of autolytic damage which begins at slaughter. The time elapsed from slaughter to viability measurements in Fry's study was about 2 hr, while in this study it was at least 6 hr.

As the proportion of viable cells decreases the FDA/phase count ratio should decrease and the trypan/phase count ratio should increase. However, the FDA/phase count ratios varied randomly with cycle number, and ratios differed significantly in only 2 of the 16 comparisons tested (Table 2). This lack of significant findings on changes in the viability of the isolated cells as measured by the uptake in FDA may result because as the number of fluorescein-containing (viable) cells decreased, the naturally fluorescent cells became more visible. Both populations of cells were counted as viable, causing random variations in FDA/phase count ratios.

Viability of the cells did decrease during F-T and R-S storage. The trypan/phase count ratio showed a significant linear increase as cycle number increased for both F-T and R-S livers (Table 2). F-T trypan/phase count ratios are significantly higher than fresh at cycles 4, 5, and 6 and R-S trypan/phase count ratios are significantly higher at all cycles (Table 2). The only significant difference between F-T and R-S trypan/phase count ratios was at 5 cycles.

R-S and F-T storage affected the viability of isolable liver cells differently. F-T treatment, while destroying some cells, preserved cellular functions related to trypan blue exclusion in others during the first two F-T cycles. By cycle 4, viability of surviving cells was significantly decreased. R-S storage, however, while not destroying the cells, caused significant decreases in viability after one cycle presumably from autolytic damage.

Increases in the amount of protein released by the PBS washes indicated a degree of structural damage to liver tissue where proteinaceous material was retained in the tissue sampled, but was easily removed by washing with PBS. The PBS protein fraction did not vary with cycle number in a linear manner for F-T livers but did for R-S livers (Table 3). F-T liver had significantly higher amounts of easily removed protein than fresh liver for all cycles but cycle 6. Damage caused by one freeze-thaw cycle was demonstrated by the increase in the amount of PBS protein isolated at one cycle. The decreasing amounts of PBS protein during extended F-T cycling indicated changes in the tissue sampled.

Damage to the liver tissue, as shown by PBS protein changes, during R-S followed a different pattern than during F-T. The increase in the PBS protein from R-S livers did not occur until cycle 4 and the maximum occurred at cycle 6. Maximum destruction of cells in R-S livers also occurred at cycle 6 suggesting that, for R-S livers, the material from the destroyed cells is removed in the PBS washes rather than contributing to the drip.

Damage to cell-cell adhesion mechanisms was demonstrated by significant linear increases in protein content of PBS-EGTA as the cycle number increased for both F-T and R-S livers. F-T livers had significantly higher amounts of

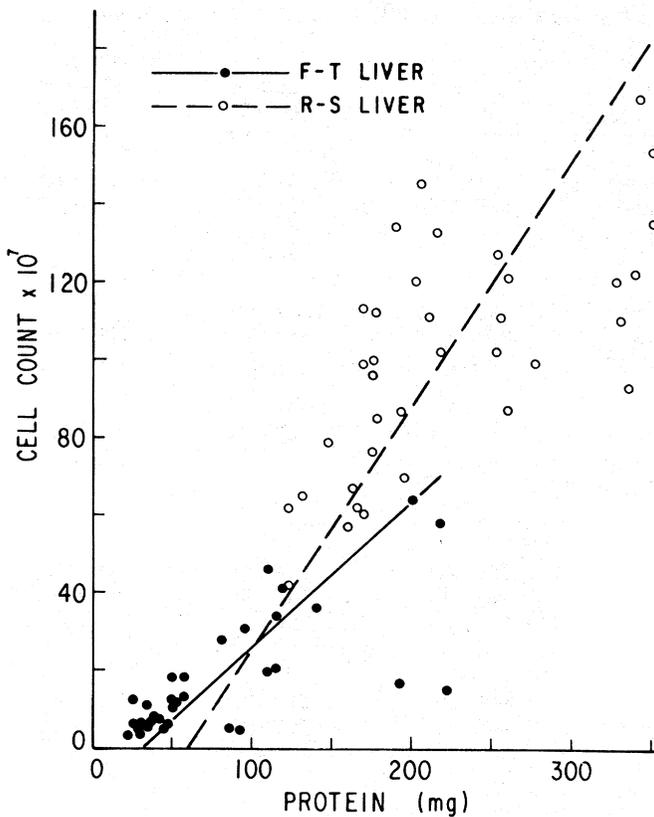


Fig. 3—Relationship between cell count and cell fraction protein content. Least squares fit for freeze-thaw liver data,  $r = 0.73$ . Least squares fit for refrigerated stored liver,  $r = 0.66$ .

protein extracted by PBS-EGTA than fresh liver throughout the storage, and R-S livers had significantly higher amounts of protein removed after cycles 4, 5, and 6 than fresh liver (Table 3). PBS-EGTA extracted significantly more protein from F-T than from R-S at cycles 1, 2, 5, and 6 (Table 3). Adhesion mechanisms were damaged either by the formation of intercellular ice and concomitant osmotic shrinkage of the cells in their supporting network of collagen, or by the mechanical stress of the intercellular ice. Partmann (1973) noted, in a histochemical study, that groups of liver cells were pressed apart by the formation of intercellular ice.

The amount of cell debris protein decreased significantly during cycling for F-T livers. However, the differences in cell debris protein between F-T and R-S livers, and fresh and R-S livers were not significant (Table 3).

The total protein recovered decreased during storage for F-T liver and remained about the same for R-S liver (Table 3). The total protein recovered in the isolation procedure was significantly lower for F-T liver than for R-S liver and for F-T liver than for fresh liver after 4, 5, and 6 cycles. Extensive damage to the cellular portion of the tissue during F-T caused an increase in the proportion of connective tissue, principally collagen (discarded as tissue debris in the cell isolation procedure), in the tissue sampled. An increase in the amount of a gelatinous substance extruded during tissue slicing was noted as the number of freeze-thaw cycles increased, and there was a gradual increase in the number of slices of tissue needed to obtain 3g of sample. This extensive destruction of cells, but not the collagen network which surrounds and supports the cells, may have an effect on the texture of the frozen liver. The effect of freezing on the texture of liver is not known. There were no significant differences when total protein recovered from fresh liver

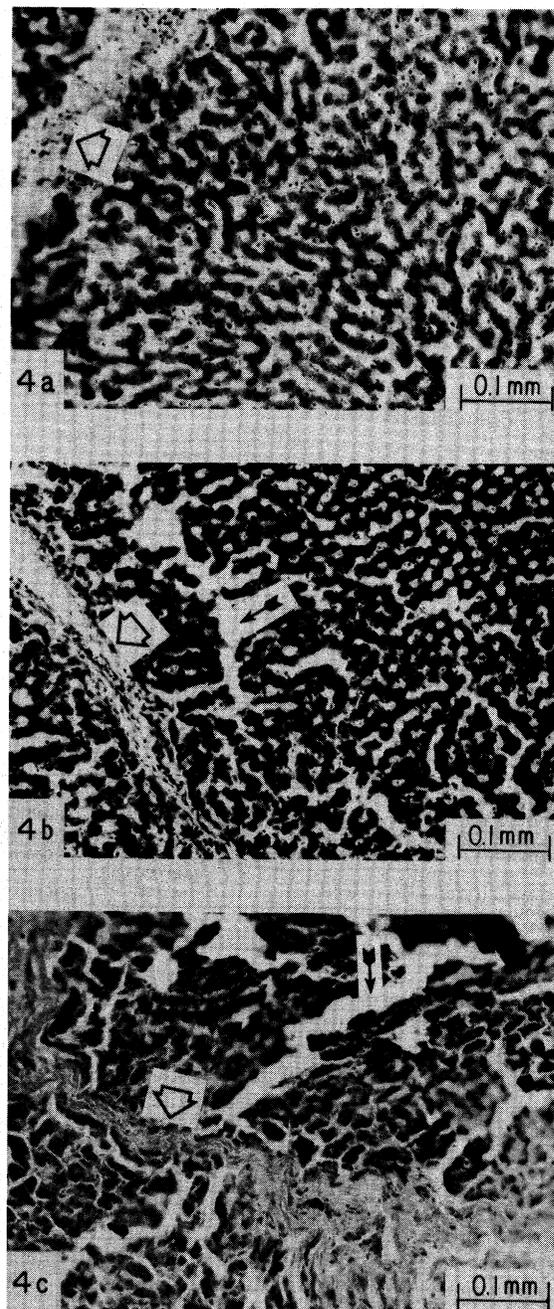


Fig. 4—Hematoxylin and eosin stained liver sections. A — Liver section, Fresh; B — Liver section, After six cycles of refrigerated storage; C — Liver section, After six freeze-thaw cycles;  $\triangle$  Collagen matrix;  $\leftarrow$  Large spaces between cells not present in fresh liver.

was compared with the total protein recovered from the R-S liver (Table 3).

Fig. 4 shows the changes in the histology of the liver during storage. The collagen cage surrounding the lobule of the liver remained intact during both R-S and F-T. The liver cells in the R-S liver were partially destroyed and the spaces between the chords of the cells were enlarged. The micrograph of the F-T liver shows much more extensive damage to the cellular portion of the liver. Large areas of the lobule had no cells present, and the cells that are present had distorted nuclei. These micrographs confirm qualitatively some of the changes in the liver quantitatively measured in this study.