Chemical and Enzymatic Changes in Liver Following Freezing-Drying and Acetone Fixation.* (19799)

M. Berenbom, H. O. Yokoyama, and R. E. Stowell.

From the Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kan.

The superior quality of frozen-dried preparations for histochemical and cytochemical localization of substances compared to chemically fixed tissues has been reported by many investigators. Theoretically, tissues prepared in this manner should show minimum changes from the original fresh tissues both chemically and structurally, except those physico-chemical alterations induced by freezing-drying itself. Whether such ideal conditions persist after paraffinization, deparaffinization, and hydration of frozen-dried tissues still needs to be investigated. Attempts at chemical analyses of frozen-dried material have been relatively few and of limited scope(1-4).

In the present investigation chemical and enzymatic assays were made on tissues in different stages of preparation in the freezing-drying procedure(5). Values from fresh control tissues were compared with those after freezing, freezing-drying, and freezing-drying followed by paraffin embedding, deparaffinization, and hydration. Further studies were made on the individual effects of heat and xylol on frozen-dried tissues. Comparative studies were also made on tissues prepared by cold acetone fixation, which is frequently used in histochemical studies. Information on quantitative effects of acetone fixation has been limited(2,6).

Materials and methods. Livers were obtained from normal strain A mice which were 12 ± 2 weeks old. Samples of tissue were taken from pooled, sliced left lateral and median lobes of 3 or 4 animals. In each experiment fresh samples weighing .4-1 g were taken. One sample was always analyzed immediately for control values, the others were sliced further into thin pieces (.5-1 mm in thickness), subjected to procedures used for histological preparations and then analyzed chemically. For freezing-drying, pieces were frozen individually in isopentane cooled by liquid nitrogen. They were dehydrated *in vacuo* at —25° to —30°C(7) over a period of 3 to 4 days. To observe the effects of freezing alone, tissues frozen as above were wrapped in aluminum foil and stored in a sealed test tube at —30°C for 3 days. The storage mixture consisted of 95% ethyl alcohol and dry ice. In experiments with paraffin embedding, frozen-dried tissues were infiltrated with paraffin at 60°C for ½ hour. To approximate the effects of heat employed in this procedure, separate samples of frozen-dried tissues were placed in a desiccator in the oven under comparable conditions. For acetone fixation pieces comparable in size to those for freezing-drying were placed in absolute acetone at 6°C for 3 days. Three changes were made on the first day and one on each of the following days. Some samples were analyzed directly after this procedure. Others were treated with 2 changes of chloroform for 1 hour at room temperature and infiltrated with paraffin as described. Paraffin blocks of frozen-dried and acetone-fixed tissues were sectioned at 25 μ thickness soon after embedding. Sections were stored overnight in sealed centrifuge tubes in the refrigerator at 4-5°C and were analyzed after deparaffinization and hydration by washing and centrifuging in the following solvents at room temperature: xylol, 30 minutes (5 changes); acetone, 30 minutes (4 changes); 70% acetone, 15 minutes (2 changes); distilled water, 15 minutes (3 changes). This schedule, necessarily longer than the usual histological procedure because of the thicker sections and larger quantities of tissue involved, was strictly followed. Additional experiments were carried out to determine the effects of xylol used in extracting the paraffin. Frozen-dried samples were homogenized with 3 successive portions of xylol, which

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TABLE I. Percentage Recovery of Chemical Constituents of Liver Following Various Treatments.

Treatment	No. of samples	Total N	Total P	Acid soluble N	Acid soluble P	Lipid soluble N	Lipid soluble P	Protein N	PNA	DNA
Freezing	5	95±1§	93±2	100±1	96±4	96±8	90±2	94±2	96±2	95±4
FD*	6–14	89±2	90±2	101±3	86±2	82±7	89±1	90±2	87±3	87±3
FD-xylol	6	89±2	86±4	105±7	98±7	86±5	82±5	86±3	88±4	90±2
FD-heat	10–14	92±2	90±2	97±4	94±4	98±5	87±3	89±2	86±1	93±3
FD-P†	6–10	69±3	29±2	25±4	14±3	36±9	28±3	83±3	26±2	80±2
Acetone	5–8	75±2	60±2	33±4	25±2	85±4	56±4	84±2	81±3	94±6
Acetone-P‡	6–10	67±3	26±2	14±2	6±1	38±6	6±2	79±3	78±4	71±3

* Freezing-drying. † Freezing-drying followed by paraffin embedding, deparaffinization, and hydration. ‡ Acetone fixation followed by paraffin embedding, deparaffinization, and hydration.
§ Stand. error. N = nitrogen; P = phosphorus.

TABLE II. Percentage of Chemical Constituents of Liver Based on Protein Nitrogen and DNA.

Treatment	Total N	Total P	Acid soluble N	Acid soluble P	Lipid soluble N	Lipid soluble P	Protein N	PNA	DNA
Freezing									
Protein N	101	99	106	102	102	96	100	102	101
DNA	100	98	105	101	101	95	99	101	100
FD									
Protein N	99	100	112	96	91	99	100	97	97
DNA	100	101	113	97	92	100	101	98	100
FD-xylol									
Protein N	103	92	122	114	100	95	100	102	104
DNA	99	95	116	109	95	91	95	97	100
FD-heat									
Protein N	103	101	109	105	110	97	100	96	104
DNA	99	97	104	101	105	94	96	92	100
FD-P									
Protein N	83	35	30	17	43	34	100	31	96
DNA	86	36	31	18	45	35	104	32	100
Acetone									
Protein N	89	71	39	30	101	67	100	96	112
DNA	80	64	35	26	90	59	89	86	100
Acetone-P									
Protein N	85	33	18	8	48	8	100	99	90
DNA	94	37	20	8	54	8	111	110	100

See Table I for explanation of abbreviations.

was finally removed by evaporation under reduced pressure at room temperature. The various samples prepared by the foregoing procedures were homogenized with cold 0.85% saline and made up to volume in a 10 ml volumetric flask. Aliquots were assayed for acid and alkaline phosphatase(8), esterase (9), succinoxidase and cytochrome c oxidase (10). In addition aliquots were fractionated according to the method of Schneider(11) and each fraction was analyzed for nitrogen (micro-Kjeldahl) or phosphorus(12) or both. Analyses were also performed for desoxypentose nucleic acid, DNA(13), and pentose nucleic acid, PNA(14).

Results. The effects of histological procedures upon the various chemical constituents of mouse liver are presented in Tables I and II. Table I shows the results in percentages of the original content, as determined from the fresh weight of the tissue and the values obtained in the control sample. In Table II an attempt has been made to correct for losses associated with manipulation of the tissue before it is ready for chemical analysis. Such losses occur because of adhesion of tissue to the razor blade in cutting a large number of small pieces, adhesion of tissue to forceps, and fragmentation of tissue during the freezing operation. This correction was made by calculating the results on the basis of recovered DNA and protein nitrogen, assuming that these 2 components would be least affected by the subsequent washing procedures employed.

TABLE III. Effect of Various Histological Procedures on Enzymes of Mouse Liver, Expressed as % of Control Based on Protein Nitrogen.

Treatment	No. of samples	Alkaline phosphatase	Acid phosphatase	Succin-oxidase	Cytochrome oxidase	Esterase
Freezing	5	110 ± 8*	87 ± 6	52 ± 9	33 ± 4	88 ± 6
FD	14	87 ± 5	92 ± 4	37 ± 4	56 ± 8	91 ± 5
FD-xylol	6	111 ± 6	108 ± 4	70 ±20	39 ± 8	96 ± 9
FD-heat	14	94 ± 5	92 ± 5	29 ± 7	58 ±11	76 ± 5
FD-P	10	29 ± 5	13 ± 6	0	0	10 ± 2
Acetone	8	49 ± 3	49 ± 6	0	0	26 ± 2
Acetone-P	10	26 ± 2	11 ± 1	0	0	11 ± 3

* Stand. error. See Table I for explanation of abbreviations.

This assumption seems to be justified since the recoveries were essentially the same when referred to these different baselines.

Freezing, freezing-drying, and freezing-drying followed by heating or xylol treatment had little or no effect on most of the nitrogen and phosphorus containing fractions of mouse liver (Table II). There was, however, a relative increase in the amount of acid soluble nitrogen, which may be due to degradation of protein during the freezing or freezing-drying process, resulting in an increase in the acid soluble material relative to the protein. The elevated content of acid soluble nitrogen relative to DNA may be explained by assuming that either DNA was incompletely extracted or that partial degradation of nucleic acid has occurred, resulting in a relatively lower DNA content in the nucleic acid fraction. Acetone fixation resulted in considerable removal of acid soluble and lipid soluble material. The effects of paraffin embedding, deparaffinization and hydration were equally severe on acetone-fixed and frozen-dried tissue, and resulted in substantial loss of acid soluble and lipid soluble nitrogen and phosphorus. There was also a significant loss of PNA in frozen-dried tissue which had been subjected to the deparaffinization-hydration process.

Freezing of tissues, freezing-drying, and freezing-drying followed by xylol or heat produced little or no effect on the acid and alkaline phosphatase or esterase but resulted in substantial loss of succinoxidase and cytochrome c oxidase activity (Table III). Freezing-drying followed by paraffin embedding and deparaffinization-hydration resulted in complete loss of succinoxidase and cytochrome c oxidase and considerable reduction in phosphatase and esterase activity. Acetone fixation completely destroyed the oxidase activity and caused a marked decrease in the activity of the other enzymes.

Discussion. Liver tissue prepared by the 2 principal methods now employed for microscopic demonstration of enzymes has been analyzed quantitatively for various chemical constituents and enzyme activity at different stages of preparation. The results obtained confirm previous claims that little change occurs in the chemical composition of frozen-dried tissues. Frozen-dried preparations are chemically similar to frozen controls. Of the enzymes studied the phosphatases and esterase appear to be unaffected both by freezing and freezing-drying. Doyle(2) has reported high activity for phosphatases in frozen-dried lymph nodules. The process of freezing and storage, however, is sufficient to cause a reduction in oxidase activity. This is in agreement with the results of Novikoff and Potter (15), and Nowinski and DeRobertis(3).

It is evident that in frozen-dried tissues much of the enzyme activity and soluble constituents are lost during the paraffin embedding, deparaffinization, and hydration procedure. Since neither the heat nor xylol treatment affects enzyme activity appreciably, it must be assumed that the loss of activity is associated with either the hot paraffin treatment or the repeated washing procedure subsequent to the xylol treatment, or both. Although the effects of graded alcohol series for hydration were not investigated, losses similar to those which occur during graded acetone hydration may be expected since the 2 procedures are essentially alike. The time for hydration of tissue subsequent to paraffin em-

bedding was longer in the present investigation than is normally used in routine histochemistry; this was necessary because of the larger amount of tissue used. The conditions may be comparable, however, since in the usual histochemical technics diffusion may be more rapid than in the larger pieces of tissue used here. It follows, then, that better results would be obtained in microscopic sections if this hydration sequence could be avoided. It has been observed(16) and confirmed in this laboratory that in the histochemical demonstration of acid phosphatase(17) considerably stronger reactions are obtained when non-deparaffinized sections are employed. Shorter incubation times are required and localization seems to be better. It has also been observed (18,19) that even with deparaffinized tissues, the microscopic appearance of frozen-dried tissues is superior to that of acetone-fixed tissues, although quantitatively both materials are equivalent in enzymatic activity (Table III).

The data presented here show that the value of freezing-drying for histochemistry may be diminished by the subsequent treatment of the tissues. It is desirable therefore to improve the methods of handling frozen-dried material. Emphasis should be placed on technics which avoid excessive contact of the tissues with solvents. Staining and histochemical enzyme procedures which require relatively short periods of immersion in aqueous solutions should also be preferable for retaining various substances. The use of carbowax(7,20) has been suggested.

Summary. 1. The chemical composition and enzymatic activity of mouse liver following procedures used in the preparation of tissues for histochemical studies show that high percentages of enzyme activity and chemical constituents are retained after freezing alone and freezing-drying. Negligible additional losses occurred in frozen-dried tissues as a result of a short heating period and treatment with xylol. Considerable loss of material and substantial reduction in the activity of acid and alkaline phosphatase and esterase occur after paraffin embedding, deparaffinization and hydration. Under the same conditions the activity of succinoxidase and cytochrome c oxidase is completely destroyed. 2. In acetone fixation, in contrast to freezing-drying, there is a greater initial loss of enzyme activity and soluble substances, with further loss upon deparaffinization and hydration.

1. Bensley, R. R., and Hoerr, N. L., *Anat. Rec.,* 1934, v60, 251.

2. Doyle, W. L., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 43.

3. Nowinski, W. W., and De Robertis, E., *Rev. Soc. argent. de biol.,* 1943, v19, 527.

4. Sylven, B., *Acta Union Internat. Contre le Cancer,* 1951, v7, 708.

5. Yokoyama, H. O., Berenbom, M., and Stowell, R. E., *Proc. Histochemical Soc.,* 1952, *J. Nat. Cancer Inst.,* in press.

6. Stafford, R. O., and Atkinson, W. B., *Science,* 1948, v107, 279.

7. Stowell, R. E., *Stain Technol.,* 1951, v26, 105.

8. Tsuboi, K. K., *Biochim. Biophys. Acta,* 1952, v8, 173.

9. Omachi, A., Barnum, C. P., and Glick, D., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 133.

10. Schneider, W. C., and Potter, Van R., *J. Biol. Chem.,* 1943, v149, 217.

11. Schneider, W. C., *J. Biol. Chem.,* 1945, v161, 293.

12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.

13. Dische, Z., *Mikrochemie,* 1930, v8, 4.

14. Brown, H., *Arch. Biochem.,* 1946, v11, 269.

15. Novikoff, A. B., and Potter, Van R., *J. Biol. Chem.,* 1948, v173, 223.

16. Goetsch, J. B., and Reynolds, P. M., *Stain Technol.,* 1951, v26, 145.

17. Gomori, G., *Stain Technol.,* 1950, v25, 81.

18. Emmel, M., *Anat. Rec.,* 1946, v95, 159.

19. Yokoyama, H. O., and Stowell, R. E., *J. Nat. Cancer Inst.,* 1951, v12, 211.

20. Firminger, H. I., *Stain Technol.,* 1950, v25, 121.

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