Preservation of Integrity of Rat Tissues for Cytochemical Staining Purposes.* By S. J. Holt, Eluned E. Hobbiger, and G. L. S. Pawan. (*From the Courtauld Institute of Biochemistry and the Department of Medicine, Middlesex Hospital Medical School, London.*)‡

The use of sucrose solutions as suspending media for preparation of tissue homogenates and subsequent centrifugal fractionation has become a widely accepted procedure. Recently, it has been claimed that addition of colloids to the medium results in better preservation of the morphological and enzymic integrity of the isolated fractions (4, 8, 15). Such media also have valuable applications during the preparation of tissues for cytochemical staining procedures.

Brief mention has already been made elsewhere (11, 13) of the value of cold formol-calcium fixation, followed by impregnation with 0.88 M sucrose containing 1 per cent gum acacia ("gum-sucrose') at 0–2°C., for the preservation of esterases in tissues, and for facilitating the cutting of thin frozen sections. Results are now described which indicate that this procedure affords better preservation of esterases, acid phosphatase, and phospholipid than certain alternative procedures currently used for staining experiments, and that better morphological preservation is also obtained, as judged by the light microscope. In addition, enzymic activity can be preserved for very long periods when the fixed tissues are stored in cold gum-sucrose.

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The improvement in sectioning arises from the fact that the imbibed sucrose medium freezes to a wax-like supporting solid, in which crystallization and concomitant cytological damage is reduced to a minimum. Dextran, polyvinyl alcohol, or polyvinylpyrrolidone may replace gum acacia in this respect, but have no apparent advantage over the natural gum.

Materials and Methods

Twelve male Wistar rats were each killed by a blow on the head and livers and kidneys were rapidly removed and chilled, and cut into 2 mm. slices using a mechanical cutting guide. The area of each slice did not exceed 1 cm.^{2}. One slice of liver from each rat was taken, and the pooled slices were weighed and transferred to fixatives (100 v/w) at 0–2°C. Fixatives were formol-calcium (4 per cent w/v formaldehyde containing 1 per cent w/v calcium chloride), and acetone. Formalin-fixed tissues were (*a*) blotted, (*b*) washed in running water for 12 hours, (*c*) blotted and placed in gum-sucrose at 0–2°C. for periods up to 2 years, (*d*) embedded by successive 30 minute treatments in 50, 70, 90 per cent, and absolute alcohols (2 changes of the latter), toluene, and 2 changes of paraffin wax (m.p. 45°C.). Embedding of acetone-fixed tissues started at the toluene stage of this procedure. Kidney slices were treated in an identical way. In all cases, similar pooled and weighed untreated tissues were used for control determinations. This procedure gave sufficient tissue for all the determinations described below to be made in parallel.

Weighed pieces (about 2 mg.) of each organ were also frozen-dried for 48 hours at −40°C. and 10^{-2} mm. pressure after quenching in a liquid propane: *iso*pentane mixture (3:1 v/v) cooled in liquid nitrogen. They were then embedded *in vacuo* for 5 minutes in 45°C. wax.

All wax-embedded tissues were dewaxed by stirring in 100 volumes benzene (4 changes of 30 minutes) at room temperature before the determinations described below were made.

Analytical Determinations

Determinations of enzymic activity were made on 1 to 10 per cent homogenates of the tissues in 0.25 M sucrose, prepared by 5 to 10 minutes treatment in an ice-cooled glass Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenates were then frozen and thawed 6 times to ensure complete activation of acid phosphatase (7). Esterase activity was determined manometrically as described previously (10) using indoxyl acetate as substrate, so that the results would also be relevant to indigogenic methods for localization of cellular esterases (11, 13). Acid phosphatase was determined using phenyl phosphate as substrate (9), and in some cases by the method of Berthet and de Duve (3), except that cacodylate buffer was replaced by 0.05 M acetate buffer. Phospholipid determinations were made on liver samples only. These were ground with sand and then exhaustively extracted, first with 3 changes of boiling chloroform-methanol (6), and then with methylal-methanol (5). After removal of solvent from the pooled extracts of each sample, lipid phosphorus was determined on the residue by the method of King (14).

Results are given in Table I and are expressed as percentages of the values for the same weights of untreated control tissues.

It will be seen that the highest esterase and acid phosphatase activities are given by formol-calcium fixed tissues impregnated with gum-sucrose for 7 to 30 days at 0–2°C., and that even after 2 years storage in this way, the phosphatase activity is still greater than that of water-washed tissues or of either type of paraffin-embedded tissues. It should also be noted that all the enzymic activities refer to tissues which would merely have to be sectioned to bring them into a state suitable for staining. They therefore have a direct practical comparative significance. The high activity of the gum-sucrose impregnated material appears to be maintained during certain staining procedures, for when sections were preincubated in substrate-free staining solutions for esterase (11) or acid phosphatase (12) for a period equal to the normal staining time, and then subsequently stained in the complete media, staining intensities were indistinguishable from those of control sections that had been stained directly.

The highest phospholipid level is found in the formol-calcium fixed liver, confirming the efficacy of the fixative (1) in this respect also. In general, a high phospholipid level is accompanied by high enzymic activity, and *vice versa*. With formol-calcium fixed tissues that were merely blotted, however, the low measured activities must be due to absorbed formalin acting upon the enzymes during homogenization. This was indicated by adding formalin to homogenates of fresh liver so that the relative amounts of tissue and formalin corresponded to those obtaining during fixation. Although quantitative determinations were made difficult by the continuing action of the formalin, a very rapid loss of the enzymes occurred, amount-

TABLE I

Tissue treatment		Kidney		Liver		
Fixation	After treatment	Acid phosphatase	Esterase	Acid phosphatase	Esterase	Phospholipid
None	None	100	100	100	100	100
Formol-calcium 2°C./24 hrs.	Blotted	8.0 (7)	20	8.5 (9)	24	96
Formol-calcium 2°C./24 hrs.	Washed 12 hrs.	41	42	38	39	94
Formol-calcium 2°C./24 hrs.	Gum-sucrose 2°C./24 hrs.	43	48	40	52	—
Formol-calcium 2°C./24 hrs.	Gum-sucrose 2°C./7 days	57 (60)	62	55 (57)	55	96
Formol-calcium 2°C./24 hrs.	Gum-sucrose 2°C./30 days	57	56	55	50	—
Formol-calcium 2°C./24 hrs.	Gum-sucrose 2°C./2 yrs.	43	33	42	34	—
Formol-calcium 2°C./24 hrs.	Into paraffin, then dewaxed	1.8	4.6	1.7	7.0	29
Acetone 2°C./24 hrs.	—	76	70	74	62	23
Acetone 2°C./24 hrs.	Into paraffin, then dewaxed	20 (21)	30	28 (27)	26	16
Frozen-dried, into paraffin, then dewaxed.		98	99	97	99	—

Phosphatase figures in brackets refer to β-glycerophosphate substrate (2).

ing to about 75 per cent in about 5 minutes. It is interesting, therefore, that formalin causes relatively little destruction of the enzymes in *intact* cells during 24 hours exposure if excess fixative is subsequently washed out under hypertonic conditions by gum-sucrose. In the case of acetone-fixed unembedded tissues, the high activity present, in spite of low retention of phospholipid, is undoubtedly due to precipitation of the enzyme under the anhydrous conditions. However, the enzyme is, in fact, rendered labile by acetone, and is subject to diffusion when exposed to aqueous media, as discussed fully elsewhere (12).

Preliminary results show that about 80 to 90 per cent of the cholinesterase activities of rat kidney and liver are also preserved by formol-calcium fixation and gum-sucrose impregnation. Satisfactory reproducibility of results was not obtained, however, probably due to the very low initial activity of the enzyme in these tissues. It is hoped to obtain reliable results with other tissues having higher levels of activity.

The method is unsuitable for alkaline phosphatase, for although initial activities are high, rapid loss of the enzyme occurs in gum-sucrose.

Although the frozen-dried tissues exhibited activities very close to those of fresh tissues, subsequent fixation of dewaxed sections, *e.g.* by exposure to formalin vapour for 30 seconds, caused a large loss of activity, for rates of staining in both esterase and acid phosphatase substrates were much slower than those of frozen sections of gum-sucrose treated formol-calcium–fixed tissues. The same localizations were given by both types of tissue, however.

The accompanying illustrations are of variously treated sections of rat kidney cortex. The excellent cytological preservation of gum-sucrose-treated formol-calcium–fixed tissues is shown in Fig. 1, which is a phase contrast photomicrograph of a

2.5 μ frozen section (× 1750). For comparison, the effects of acetone fixation are shown in the equivalent micrograph of a dewaxed paraffin section (Fig. 2). Results of applying the recently re-examined (12) Gomori staining technique for acid phosphatase to the gum-sucrose impregnated sections is shown in Fig. 3 (× 750). Fig. 4 shows the results given by applying the Baker acid haematein method (1) for phospholipids to formol-calcium–fixed tissue (× 750). During this procedure, the cutting of frozen sections was greatly facilitated by impregnating the postchromed tissue with gum-sucrose for 24 hours. A nuclear counterstain has been applied to the sections shown in Figs. 3 and 4.

Although it is probable that the acid haematein stain mainly reveals relatively high concentrations of phospholipid, or that which is relatively weakly bound (*e.g.* mitochondria are only very weakly stained in Fig. 4), the correspondence between the results shown in the last two figures is of interest in view of the finding that good retention of phospholipid is accompanied by high phosphatase activity. That the acid phosphatase integrity of both rat kidney and liver depends upon maintainance of their phospholipid integrity is discussed more fully elsewhere (12), but it should be mentioned that the droplets which are so clearly stained by the acid haematein technique in Fig. 4 remain completely unstained in the phospholipid-depleted acetone-fixed kidney sections, which, in turn, give very diffuse staining patterns when treated by the Gomori acid phosphatase technique (12). Similar conclusions have been reached by Beaufay and de Duve (2) concerning the importance of intact lipoprotein barriers for maintaining the acid phosphatase activity of isolated liver lysosomes. It is also of interest that the presence of acid phosphatase in the droplets shown in Fig. 3 agrees with the findings of cell fractionation studies (16).

Demonstration of esterases in the same droplets, and a similar correspondence between the locations of esterase and acid phosphatase in rat liver have been reported previously (12, 13). The significance of these findings and of closely related results given by preliminary studies with new staining methods for other hydrolytic enzymes is being investigated more fully. Meanwhile, it appears that formol-calcium fixation and gum-sucrose impregnation provides tissues which are particularly suitable for certain correlated biochemical and cytochemical staining studies.

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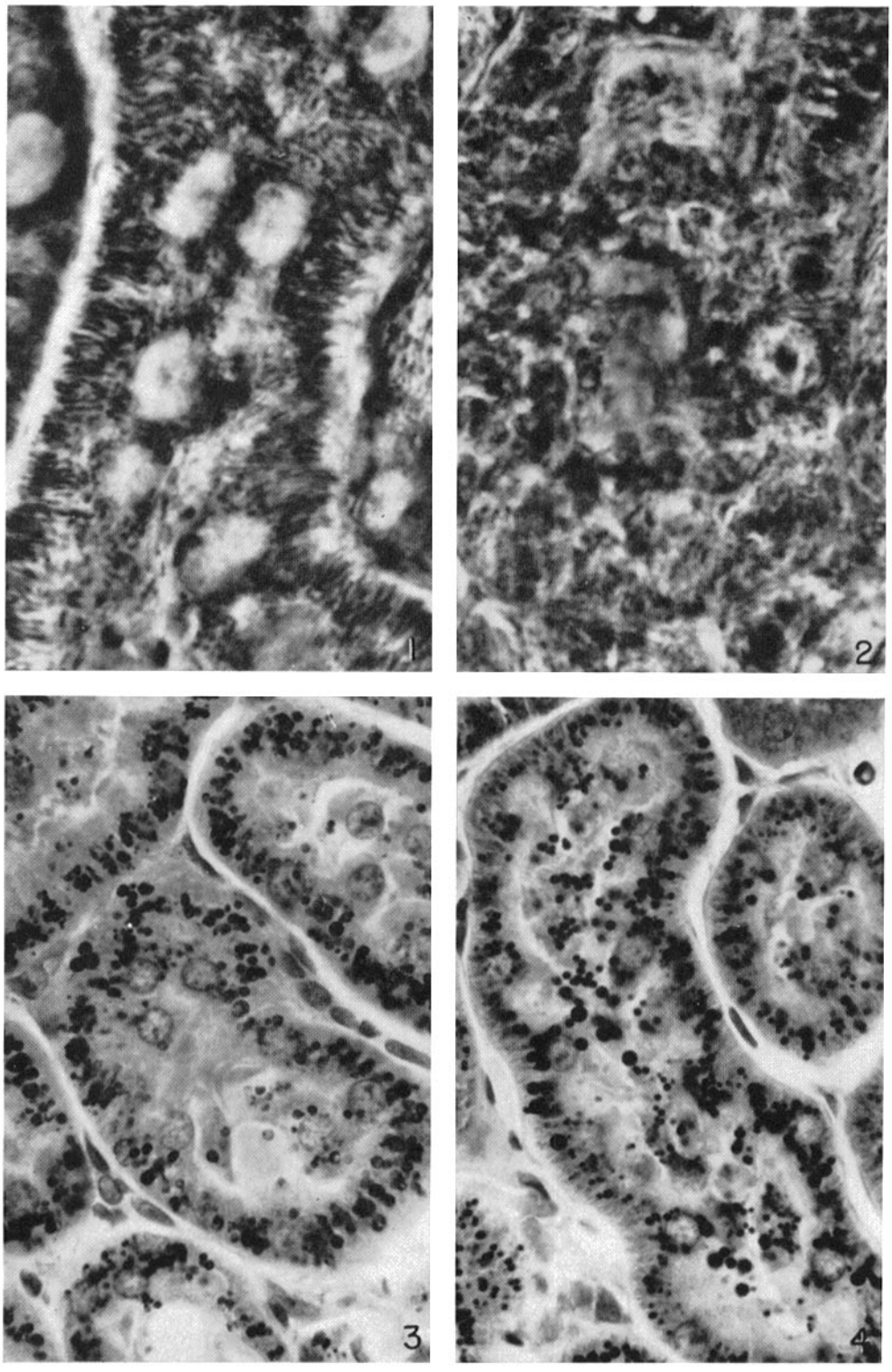
Explanation of Plate 210

Figs. 1 to 4. For description see text.



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