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Effects of Dehydrating Agents on Phosphatases in the Lymphatic Nodules of the Rabbit Appendix.

WILLIAM L. DOYLE.*

From the Department of Anatomy, University of Chicago.

In the course of a study on the quantitative correlation between phosphatase activities and histological changes in lymphatic tissue it has been necessary to determine storage properties and stability to dehydration of these enzymes in our material. The rabbit appendix was selected because it presents an almost diagrammatically homogeneous distribution of lymphatic nodules over a large area. This arrangement is ideal for histochemical examination of the effects of experimental modifications.[1] Rabbits were killed with nembutal and the appendix promptly removed, washed with saline, drained, weighed and dehydrated as indicated or minced and homogenized fresh. Strips weighing about one gram each were made up to 20 ml of homogenate. Determinations were carried out in triplicate at 30°C and 40°C with 2.5-5.0 mg of fresh weight of tissue in suspension. Disodium phenylphosphate (0.5%) was used as substrate with barbital or glycine buffers. The reaction was stopped at the end of 30 minutes by addition of Folin-Ciocalteau phenol reagent. No activation was found with added magnesium. Tissues to be dehydrated were dropped into 50 volumes of absolute acetone, 80% acetone, absolute alcohol, 80% alcohol, absolute methyl alcohol (all at room temperature) or into isopentane chilled in liquid nitrogen. The samples frozen in isopentane were dehydrated at –40°C *in vacuo*. The other samples were left in the fixative at 5°C for 24 hours and then drained and homogenized in water in a Potter-Elvehjem homogenizer.

Alkaline phosphatase. The optimum for alkaline phosphatase activity was found to be at pH 9.8 with 20% less activity at 9.6 and 10.1. Freshly homogenized appendix gave values for 6 different rabbits of 7.0 to 11.0 μg phenol liberated per milligram fresh weight of tissue in 30 minutes at 30°C. The activity at 40°C was 1.35 times as great. Single values in the triplicate determinations at each step agreed within 1%. Taking the fresh homogenate as 100% activity we found in the different specimens 95 ± 5% activity after storage of the homogenate at 5° C for 24, 48, 72 and 96 hours. The frozen dried tissue gave 85 ± 8% of the fresh value at periods of 1, 48 and 96 hours after homogenizing. After fixation in absolute methyl alcohol only 15% activity remained. When fixed for 24 hours in acetone or alcohol and then homogenized, the immediate values were as follows: absolute acetone, 80% acetone and absolute alcohol gave 75 ± 5% of the fresh activity: 80% alcohol gave 65 ± 5% activity. The acetone or alcohol fixed enzyme preparations underwent further inactivation upon storage as homogenates at 5°C at comparable steady rates so that 72 hours later about two-thirds of the initial values after fixation were obtained. Each of these fixed samples carries about 3% of the fixative into the homogenate suspension. In this material 80% alcohol has always preserved less alkaline phosphatase activity than either absolute alcohol or acetone which is at variance with the results of Stafford and Atkinson.[2] The frozen dried material has only slightly higher activity than the absolute alcohol and acetone fixed material but it is as stable upon storage as are fresh tissue homogenates. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 30% but retained full activity for two weeks when stored at –20°C.

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1 De Bruyn, P. P. H., *Anat. Rec.*, 1948, **101**, in press.

2 Stafford, R. O., and Atkinson, W. B., *Science*, 1947, **107**, 297.

Acid phosphatase. Maximal acid phosphatase activity was obtained at pH 5.6 - 5.8 with about 10% less activity at 5.4 and 6.2. The freshly homogenized tissues gave individual values for the same 6 rabbits ranging from 10 to 14 μg phenol liberated per mg fresh weight of tissue in 30 minutes at 30°C. Taking the value one hour after homogenizing as 100% activity we found upon storage at 5°C: 75% activity after 24 hours, 70% after 48 hours, and 55% after 96 hours. Acetone (80% or 100%) preserved 25% of the fresh activity and upon storage of the homogenate for 72 hours this decreased to 15%. Alcohol (80% or 100%) preserved 15% of the fresh activity and upon storage as homogenate this declined rapidly (24 hours) to 2%. Methyl alcohol left negligible activity. The frozen dried tissue gave 85% of the fresh activity and after 48 hours had 70% of the initial fresh value which was the same as that of the fresh material after storage for the same period. Frozen dried material stored dry in a desiccator at 25°C for 3 days showed a decrease of activity to about 15% but was stable at −20°C.

Effect of temperature of fixation. In a subsequent series of experiments with 4 rabbits it was found that for acetone fixed material distinctly better cytological fixation and better preservation of both alkaline and acid phosphatase was obtained when tissues were dropped into acetone at −20°C and stored at this temperature for 48 hours.

Effect of embedding in paraffin. A sample of acetone fixed material with an activity of 9.3 μg phenol per mg fresh weight at pH 5.6 and 8.4 μg phenol per mg fresh weight at pH 9.8 was transferred to benzene and then to paraffin at 60°C for 1 hour. A paraffin block was made and stored 24 hours at 5°C. Most of the paraffin was cut away and the small block extracted with carbon disulfide at 40°C in a Soxhlet apparatus. When homogenized in water this material showed 60% of the acid phosphatase activity of the original acetone fixed material and 55% of the alkaline phosphatase activity. Analyses of sections in which the weight of the section was estimated from its calculated volume is consistent with these findings. In the work on sections it was found that the phosphatases were stable in the paraffin block even at room temperature for several days but that sections in paraffin ribbons deteriorated at room temperature although they were stable for a week at −20°C.

Summary and Conclusions. Alkaline phosphatase in the rabbit appendix is almost as well preserved by fixation in chilled acetone as by the freezing drying technic. Acid phosphatase on the other hand is distinctly better preserved by dehydration in the frozen state than after acetone fixation. Acid phosphatase activities are maximal when determined promptly on fresh homogenates. Frozen tissues dehydrated in the frozen state and stored dry at −20°C gave acid phosphatase activities equal to 85% of fresh values and for homogenates stored at 5°C for 24-96 hours the values of fresh and frozen preparations were identical. Acetone and alcohol fixation gave marked reduction in acid phosphatase activity. Alkaline phosphatase activity of fresh tissue is reduced to about 75% of the fresh value by treatment with acetone or absolute alcohol at room temperature. The effects of temperature of fixation, storage in the dry state and embedding in paraffin are noted.