TABLE 1

| Hydrolysis in fresh tissue | To paraffin | | Through paraffin | |
|---|---|---|---|---|
| | Hydrolysis | % lost | Hydrolysis | % lost |
| 0.915 (1.68–0.37) | 0.54 (0.97–0.24) | 38.8 | 0.22 (0.38–0.08) | 76.0 |

The tissue blocks subjected to the various steps of fixation and embedding were then homogenized and extracted with 30% glycerine. The residual enzyme was determined by the microtitrametric method of Glick (*2*), using acetylcholine as the substrate in a concentration of 0.51%. Total hydrolysis was determined on 3 separate aliquots of extract from each tissue segment, yielding 39 determinations for each group of treated segments, *a*, *b*, and *c*. These data have been summarized (Table 1) by averaging all determinations, but the ranges are indicated by the bracketed figures. The highest values were obtained for the medulla and thence, in descending order, the lumbosacral enlargement, cervical, and thoracic cord. The standard error for all determintions proved to be 0.042. It may be noted here that extracts of nerve tissue and hemolyzed red cells hydrolyzed both myristoyl choline, as used in the histochemical technique (*1*), and acetylcholine in an essentially similar graphical configuration at varying but equivalent substrate concentrations. Thus, peak activities for both substrates were obtained at similar concentrations, and a parallel reduction in activity because of higher concentrations occurred for the 2 substrates. However, the total hydrolysis of myristoyl choline was only 54.2% that obtained for acetylcholine. We have felt justified in using the latter substrate to take advantage of this heightened total hydrolysis and reduce the potential error accordingly. The units of enzyme activity are expressed in μl of 0.1*N* NaOH/mg of tissue. For purposes of comparison, the enzyme concentration determined for the fresh, untreated segments was taken as 100% activity, and the loss expressed as the per cent activity remaining.

The process of fixation and dehydration destroys or inactivates about 38% of the enzyme present (Table 1), and paraffin embedding reduces enzyme activity an additional 38%. Thus three-fourths of the normal tissue enzyme activity is destroyed as a result of the rigors of fixation, dehydration, and embedding. This figure is not unlike previous reports of the loss of non-specific esterases (*3*) and phosphatases (*4*), wherein 60–95% loss in the respective enzymes was encountered in paraffin-embedded tissues. It would thus seem that consistently high enzyme losses for the esterases are to be expected in the preparation of paraffin-embedded tissues. Since the process of embedding is primarily concerned with the chemically inert substance paraffin, it is assumed that the major loss is due to the relatively high temperatures encountered. It may be recalled (*5*) that choline esterase is quite thermolabile, being rapidly inactivated at temperatures approaching 60° C.

Noting the high loss of enzyme activity in paraffin-embedded tissues, we found it possible to ascertain roughly the lower limits of enzyme concentration that must remain in the tissue in order to secure a positive histochemical reaction. In a series of experiments to be reported elsewhere, the cervical sympathetic cord was transected and the choline esterase activity in the superior ganglion determined by both histochemical and quantitative methods. Within 3 days postoperatively the histochemical reactions were negative, presumably because of loss of the enzyme in the degenerated terminal endings of the preganglionic neurons. However, quantitative determinations on these ganglia showed only a 10–15% loss of enzyme. To explain the negative histochemical results, it is presumed that the initial loss of enzyme (10%), together with the technical loss in preparing the sections (70%), results in some 20% of residual activity remaining as compared with the normal tissue, an amount insufficient to accommodate the necessary hydrolysis of the substrate to permit histochemical localization. There are undoubtedly other factors that further serve to limit the sensitivity of this reaction, but certainly the above evidence suggests that a negative histochemical reaction does not necessarily mean an absence of the enzyme, and, correspondingly, due caution should be exercised in interpreting this type of preparation. The value of making simultaneous quantitative studies, wherever the nature of the tissue elements permits, is self-evident.

### References


1. Gomori, G. *Proc. Soc. Exp. Biol. Med.*, **68**, 354 (1948).
2. Glick, D. *Techniques of Histo- and Cytochemistry.* New York: Interscience, 1949.
3. Nachlas, M. M., and Seligman, A. M. *J. Nat. Cancer Inst.*, **9**, 415 (1949).
4. Stafford, R. O., and Atkinson, W. B. *Science*, **107**, 279 (1948).
5. Allens, G. A., and Hawes, R. C. *J. Biol. Chem.*, **133**, 375 (1940).


## A Technique for Whole Mount Autoradiographs of Rabbit Mammary Glands[1,2]


**Walter S. Lundahl, Joseph Meites, and L. F. Wolterink**

***Department of Physiology and Pharmacology, Michigan State College, East Lansing***


We have observed that growing mammary glands of rabbits and rats readily take up radioactive phosphorus ($P^{32}$). By the use of the autoradiographic technique described here, the extent of mammary proliferation, as well as the regions of most active growth, can be clearly differentiated.

Autoradiographs of the mammary glands of male and female rabbits were prepared by the following procedure:


[1] Published with the approval of the Director, Michigan Agricultural Experiment Station, as Journal Article No. 1159.

[2] This study was aided in part by a grant from the U. S. Atomic Energy Commission and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

A single intravenous injection of 50 μc of $P^{32}$ per kg of body weight was given, and the rabbits were sacrificed 4 hr later. A midline incision was made into the skin over the ventral surface, care being taken not to injure the mammary tissue underneath. The whole mammary glands were carefully dissected out with a sharp razor blade, washed in cold running water to remove adhering hair or blood, and stretched out flat on plywood or cork board with the aid of thumbtacks. The whole mount was then permitted to dry thoroughly under a heat lamp for 8–24 hr. Removal of tissue exudates was facilitated by frequent blotting. Such whole mammary gland mounts were found to require no further treatment and were sufficiently thin so that sectioning was unnecessary.

Autoradiographs were prepared approximately 24 hr after sacrificing the rabbits. A sheet of pliofilm was placed between the dried mammary tissue and the emulsion side of Eastman x-ray no-screen film. Good contact was obtained by inserting a square portion of heavy blotting paper over the tissue and holding the entire preparation in place between plywood by clamps or weights. Exposure time was 72 hr.

Counts were made of some of the mammary tissue 24 hr after sacrificing the animals. One-half-inch circular discs, weighing approximately 132 mg, were cut from areas close to the nipples of the glands and counted under a thin mica end-window counter. These averaged 15 cps, or about 0.01 μcuries per disc.

Fig. 1 shows an autoradiograph of a growing mammary gland taken from a male rabbit. This gland had been developed by injecting 0.1 μg of estrone and 1.0 mg of progesterone daily for 35 days. The radioactive phosphorus was injected on the 35th day, and the animal was sacrificed 4 hr later. It can be seen that most of the $P^{32}$ is localized in the peripheral area and in the nipple, or in the regions of maximal growth in the gland. The lobule-alveolar system is well developed.

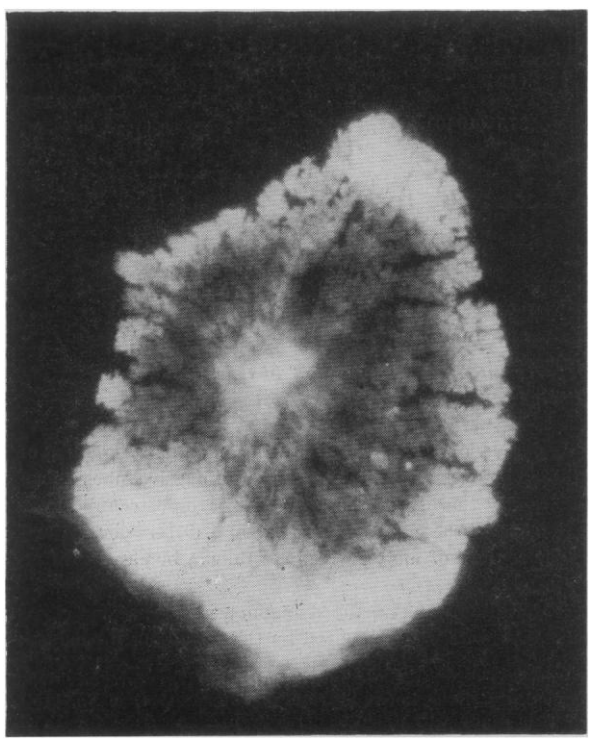

FIG. 1. Autoradiograph showing distribution of $P^{32}$ in a growing mammary gland from a male rabbit.

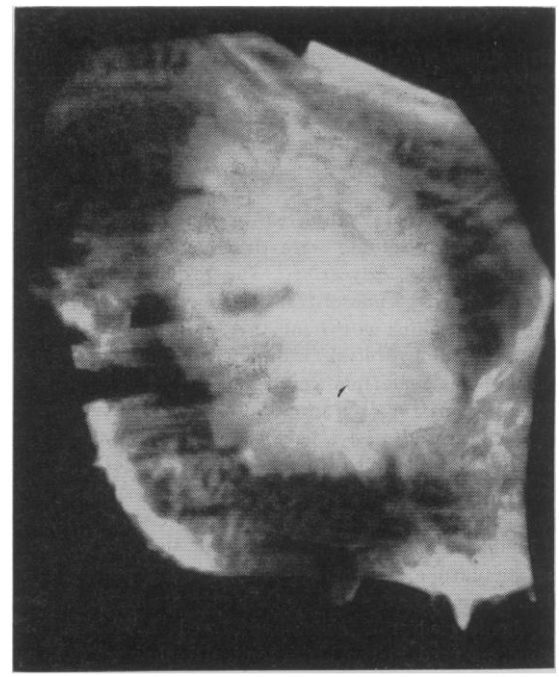

FIG. 2. Autoradiograph showing distribution of $P^{32}$ in a nongrowing, atrophic mammary gland from a male rabbit.

Fig. 2 is a radioautograph taken from a nonproliferating, atrophic mammary gland of a male rabbit. Mammary growth had been induced by injecting estrone and progesterone for 25 days. Hormone administration was then stopped for the following 10 days, and on the 35th day $P^{32}$ was injected. It can be seen that, with the exception of an area near the center of the gland, the $P^{32}$ is distributed uniformly. Unlike the growing gland, the radioactive phosphorus is not concentrated in the peripheral area or in the nipple.

These radioautographs are believed to represent mainly water-insoluble, organically bound phosphate. In a few rabbits that had been treated with lactogenic hormone, milk could be expressed from the glands, but the milk carried very little activity. Since milk normally has considerably more inorganic phosphorus than is found in blood plasma, there could have been very little phosphate in the intercellular compartment of these mammary glands. Lipid materials extracted with absolute alcohol or xylol likewise were relatively inactive, as were tissue exudates removed from the glands when they were drying under the heat lamp. Thus the bulk of the activity, insofar as these preliminary results indicate, was probably present in other organic linkages.