

EFFECTS OF FIXATION AND DEMINERALIZATION ON THE INTENSITY OF AUTORADIOGRAPHIC LABELLING OVER THE PERIODONTAL LIGAMENT OF THE MOUSE INCISOR AFTER ADMINISTRATION OF [³H]-PROLINE

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Summary—The effect of different histological procedures on the autoradiographic grain count over the periodontal ligament was studied quantitatively in autoradiographs made eight hours after administration of [³H]-proline. The lower jaws of 9 mice were fixed in Bouin's fixative, in 10 per cent formalin or in Carnoy's fluid and were demineralized either in EDTA or in a mixture of formic acid and sodium citrate. With Bouin's fixative, higher grain densities were obtained than with formalin or Carnoy's fluid. Liquid scintillation counting revealed that in formalin traces of non-dialyzable tritiated substances were present. A greater loss of non-dialyzable radioactivity occurred during the demineralization of the jaws fixed in Carnoy's fluid particularly when this was performed in a formic acid and sodium citrate mixture. For the latter mandibles, autoradiography revealed a low grain density over the periodontal ligament while the relative shrinkage of the tissue appeared to be high. Biochemical analysis of the dialysates of the formic acid and sodium citrate mixtures from these mandibles showed that part of the radioactivity lost was bound to collagen.

INTRODUCTION

In autoradiographic studies, the concentration of radioactive material in a tissue is not merely determined by biological principles but it is also influenced by the mode of histological processing. During fixation, proteins are denatured and made more or less insoluble. If fixation is incomplete, proteins and other substances may be extracted during fixation and the subsequent preparative procedures (e.g. Merriam, 1958; Burgos, Vitale-Calpe and Téllez de Inon, 1967; Schneider and Schneider, 1967; Vanha-Perttula and Grimley, 1970). This may result in a loss of incorporated labelled precursors. The degree of protein loss depends on the nature of the tissue involved (Merriam, 1958), the fixative used and the conditions of fixation such as duration, concentration of the components of the fixative and the solvents used (Flitney, 1966; Burgos *et al.*, 1967; Vanha-Perttula and Grimley, 1970).

In autoradiographic studies of protein metabolism in the periodontal ligament, many fixatives and demineralizing agents have been applied. No information is available in the literature, however, concerning the influence of the various histological procedures on the intensity of the autoradiographic labelling in that tissue after administration of a radioactive protein precursor. The purpose of the present study was to determine the possible effects of different techniques of tissue fixation and demineralization on the autoradiographic grain count over the periodontal ligament

of the lower incisor of the mouse after administration of [³H]-proline.

MATERIALS AND METHODS

Tritiated L-proline (Radiochemical Centre, Amersham, England; specific activity 1 Ci/mM, generally labelled) was administered to 9 female CBA-mice, 5-months-old, weighing 25–31 g. Each mouse received a single intraperitoneal injection of 5 µCi/g body weight. All animals were killed eight hours after injection. The lower jaws were dissected free from the surrounding soft tissues and were divided into two halves. The 9 pairs of mandibles were processed using 6 histological procedures in such a way that, for each preparation method, 3 observations could be made (Fig. 1). The mandibles were fixed using one of the following procedures (Romeis, 1968):

Carnoy's fluid (13–15 hr) at 4°C, ethanol abs. (3 hr), 70 per cent ethanol (24 hr);

10 per cent neutral buffered formalin (pH 6.9) for 48 hr at room temperature;

Bouin's fixative (24 hr) at room temperature, 70 per cent ethanol (24 hr).

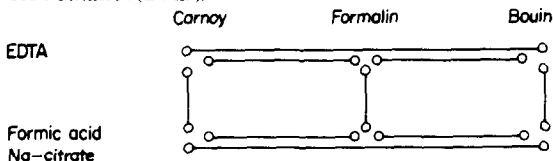


Fig. 1.

The jaws were demineralized either in 12.5 per cent EDTA (pH 7.2) for 4 days or in a mixture of formic acid and sodium citrate for 2 days (90 per cent formic acid 150 ml, tri-sodium-citrate.2 H₂O 254 g, distilled water to make 300 ml). During tissue processing, the volumes of the preparation fluids were kept constant. After fixation and demineralization, all mandibles were processed identically and simultaneously. They were double-embedded in celloidin and in Paraplast (Sherwood Medical Ind. Ltd, Crawley, Sussex, England) and were sectioned parallel to the longitudinal axes of the incisors at 5 µm.

Autoradiography

It is well known that the amount that tissues shrink depends on the technique of histological preparation. This means that the concentration of radioactive material per unit volume of tissue might be influenced. Based on the assumption that during tissue shrinkage the changes in cell density is proportional to the volumetric changes of the intercellular matrix, the autoradiographic grain count was related to the number of fibroblast nuclei per area of tissue.

From the midsagittal region of the incisors, every second section was selected to exclude the risk of cells being counted more than once. Using Kodak AR10 stripping film, 4 autoradiographs per mandible were prepared and stored in light-tight Clay Adams boxes in the presence of silica gel. They were exposed for 49 days at 4°C. Control sections of jaws from non-labelled animals, which were processed identically and simultaneously with the labelled jaws, were included to check for both latent image fading and chemography (Rogers, 1967). The autoradiographs were developed all at the same time in Kodak D19 for 5 min at 20°C, fixed in 30 per cent sodium thiosulphate for 10 min and subsequently stained with Ehrlich's acid haematoxylin.

Using an eyepiece grid, grain counts were made in the middle zone of the lingual part of the periodontal ligament of the incisor at the level of the first molar at a magnification of × 800 (Beertsen, 1973). In each section, 5 areas of 130 µm length and 6.5 µm width were subdivided into squares with a side of 6.5 µm; grain counts were made using the small square as a basic unit. Averages were derived from the counts over the 4 sections after correction for background.

The number of fibroblast nuclei per sectional area was determined as follows. In the middle zone of the periodontal ligament of the incisor, nuclei were

counted at the level of the first and second mandibular molars in 4–8 sections per mandible stained with Ehrlich's acid haematoxylin or with Feulgen's stain. For each mandible, counts were made in 25 areas of 90 µm length and 20 µm width. At the outlines of the counting areas only those nuclei were included of which at least half was situated within these areas. Small and irregularly shaped fragments were disregarded.

As a measure for the grain density over the middle zone of the ligament, the quotient between the mean autoradiographic grain count per 42.25 µm² and the mean number of fibroblast nuclei per 1800 µm² was calculated.

Liquid scintillation counting and biochemical determinations

The fixing fluids and demineralizing agents from each jaw were dialyzed against several changes of 0.1 M acetic acid in the cold for 48 hr. Before dialyzing the Carnoy's fluid, chloroform was removed by evaporation. Samples of 0.5 ml from each dialysate were added to 9.5 ml scintillation fluid consisting of a toluene solution of PPO and POPOP and 7 parts of Triton-X-100. The radioactivity was determined using a Mark II Nuclear Chicago Scintillation Spectrometer with 37 per cent counting efficiency for tritium. The radioactivity was calculated as disintegrations per minute (DPM).

When possible, proline and hydroxyproline determinations were made in the dialysates. After hydrolyzing in 6 N HCl, the hydroxyproline content was determined as described by Guis, Slootweg and Tonino (1973). For proline determination, the method of Sumner and Roszel (1965) was used. Determinations of the specific activities of hydroxyproline were performed according to M. B. Guis and G. J. M. Tonino (unpublished).

Statistical analysis

The data were statistically analyzed using Student's *t*-test. If, using the *F*-test, significant differences (2α = 0.05) appeared to be present between the variances of the samples, the *t*-test modified according to Welch (1947) as cited by De Jonge (1964) was applied.

RESULTS

The counts of fibroblast nuclei in the periodontal ligament revealed that for 5 out of the 6 preparation

Table 1. Effect of fixatives and demineralizing agents on the mean number of fibroblasts per 1800 µm² in 5 µm sections of the middle zone of the periodontal ligament (S.D.). For each treatment n = 3.

	Carnoy	formalin	Bouin
EDTA	11.68 (0.38)	12.05 (0.91)	10.51 (0.61)
	***	N.S.	*
formic acid	18.25 (0.47)	13.56 (0.08)	12.15 (0.66)
Na-citrate			

N.S. = Not significant (2α = 0.05)
* - *p* < 0.05. ** - *p* < 0.01. *** = *p* < 0.001.

Table 2. Effect of fixatives and demineralizing agents on the mean autoradiographic grain count in the middle zone of the periodontal ligament (S.D.). The counts are presented as number of Ag-grains per unit area of 6.5 to 6.5 µm and have been corrected for background. For each treatment n = 3.

	Carnoy	formalin	Bouin
EDTA	5.06 (0.28)	5.55 (0.38)	6.07 (0.44)
formic acid	4.22 (0.09)	5.20 (0.52)	5.85 (0.09)
Na-citrate			

methods the number of fibroblast nuclei per 1800 µm² varied between 10–13 (Table 1). When the jaws were fixed in Carnoy's fluid, however, and demineralized in a mixture of formic acid and sodium citrate, the counts of nuclei were relatively high when compared with the other treatments. The mean count was about 18 nuclei per 1800 µm², indicating that tissue shrinkage was more pronounced.

If demineralization was performed in EDTA, the number of fibroblast nuclei per area of tissue section appeared to be lower than for the mandibles demineralized in formic acid and sodium citrate. This was notably evident for the mandibles fixed in Carnoy's fluid (*p* < 0.001).

When the autoradiographic grain count (summarized in Table 2) was corrected for tissue shrinkage using the number of fibroblast nuclei per area of tissue section (Table 3), the grain density over the middle zone of the periodontal ligament appeared to be higher for the mandibles fixed in Bouin's fixative than for those fixed in formalin (*p* < 0.05). The grain density was lowest for the jaws fixed in Carnoy's fluid particularly when demineralization was performed in the mixture of formic acid and sodium citrate. In the latter case, the grain density appeared to be markedly less than that over the ligament of the jaws fixed either in formalin (*p* < 0.05) or in Bouin's fixative (*p* < 0.01).

When EDTA was used, the density of grains tended to be higher as compared with that for the mandibles demineralized in formic acid and sodium citrate. This tendency, however, was only significant using Carnoy's fluid as a fixative (*p* < 0.01).

The results of the liquid scintillation counting were as follows: With respect to the fixatives, traces of non-dialyzable tritiated substances were only detected in the dialysates of the formalin fixing fluid (Table 4). In the dialysates of the other fixatives, the radioactivity

Table 4. Loss of non-dialyzable tritiated substances from the lower jaws during fixation and demineralization. The results are given only for the treatments which led to measurable extraction of ³H-activity.

	n	mean loss of DPM per mandible (S.D.).
formalin	6	280 (165)
EDTA after fixation in Carnoy	3	490 (26)
formic acid-Na citrate after fixation in Carnoy	3	1770 (509)

* = *p* < 0.05. ** = *p* < 0.001.

was below background. As regards the demineralizing agents, significant levels of tritium activity were found in the dialysates of those used for the mandibles fixed in Carnoy's fluid. This was particularly evident for the formic acid and sodium citrate mixture (Table 4). After pooling the 3 dialysates of this demineralizing agent, enough material was gathered to permit determination of hydroxyproline and proline and their respective specific activities. The proline: hydroxyproline ratio was 1.6, while the proline: hydroxyproline specific radioactivity ratio was 0.44.

DISCUSSION

Our study indicates that, eight hours after administration of [³H]-proline, autoradiographic labelling occurs over the middle zone of the periodontal ligament of the mouse incisor the amount varying with the choice of the histological fixative and demineralizing agent. It should be kept in mind, however, that for correction of the relative volumetric changes of the ligament during tissue preparation an indirect method was used. The autoradiographic grain count was related to the number of fibroblast nuclei per sectional area assuming that the volumetric changes in the intercellular matrix was proportional to the changes in cell density. The results of this study, therefore, should be considered as indicative of general trends rather than as exact recordings of relative changes.

In autoradiographs of the mandibles fixed in Bouin's fixative, higher grain densities were found over the ligament as compared with those over the ligament in

Table 3. Effect of fixatives and demineralizing agents on the ratio
mean autoradiographic grain count
mean count of fibroblast nuclei × 100 (S.D.).
For each treatment n = 3.

	Carnoy	formalin	Bouin
EDTA	43.37 (2.62)	46.11 (3.21)	58.01 (6.59)
formic acid	23.10 (0.35)	38.36 (4.05)	48.27 (2.30)
Na-citrate			

N.S. = Not Significant (2x = 0.05).
* = *p* < 0.05. ** = *p* < 0.01.

the mandibles fixed in formalin. Similar findings have been made by Kruger (1969) for ameloblasts in the enamel organ of the rat incisor. The higher grain densities might be due either to adsorption of free [^3H]-proline or to a lower degree of extraction of labelled tissue components. Binding of free amino acids to tissues may lead to artifacts in autoradiography particularly when tissue levels are high, which may be the case shortly after exposure to radioactive protein precursors. Studies by Droz and Warshawsky (1963) and by Peters and Ashley (1967) suggest that neither Bouin's fixative nor formalin can bind significant amounts of free radioactive leucine to tissues. Vanha-Perttula and Grimley (1970), however, reported that formalin can produce a non-specific time related binding of leucine to human carcinoma-cell monolayers. They also found that loss of protein may be considerable during fixation in formalin and subsequent washing. Similar results have been obtained by other workers (Merriam, 1958; Burgos *et al.*, 1967; Schneider and Schneider, 1967). Our study shows that, during fixation in formalin, loss of non-dialyzable tritiated substances can occur. When Bouin's fixative was used, however, no radioactivity was found either in the dialysates of the fixative or in those of the demineralizing agents. Although no direct measurements were obtained from the periodontal ligament and the liquid scintillation counting was confined to the fixatives and demineralizing agents, these results together with the autoradiographic data suggest that, under the conditions of this study, Bouin's fixative may lead to less extraction of labelled tissue components than formalin. This could be due to a better stabilizing of the protein skeleton which may be ascribed to the high concentration of formalin and the presence of picric acid (Droz and Warshawsky, 1963).

The grain density over the periodontal ligament of the mandibles fixed in Carnoy's fluid and demineralized in the mixture of formic acid and sodium citrate was relatively low. The observed grain density was about half of that in the mandibles fixed in Carnoy's fluid and demineralized in EDTA. This suggests that radioactivity was lost as a result of the treatment with the acid demineralizing agent. This view is supported by the results of the liquid scintillation counting which showed that during demineralization with the formic acid and sodium citrate mixture loss of non-dialyzable radioactivity occurred. The finding that the relative shrinkage of the ligament appeared to be high further suggests that Carnoy's fluid does not stabilize the protein skeleton very well and renders it susceptible to extraction of proline. Biochemical analysis of the pooled dialysates of the formic acid and sodium citrate mixtures revealed a proline:hydroxyproline ratio of 1:6. As in collagen this ratio is about 1:2, the observed excess of proline indicates that, apart from collagen, non-collagenous proteins were also extracted. The proline:hydroxyproline specific-radioactivity ratio (0.44) in these dialysates, however, was much lower than the ratio, close to unity, usually found for collagen (Barnes *et al.*, 1970). This indicates that the extracted non-collagenous proteins contain mainly non-radioactive proline and that the greater part of the radioactivity lost in the formic acid and sodium citrate mixture was bound to collagen.

Carneiro and Leblond (1966), using purified bacterial collagenase to differentiate in tissue sections

between collagen and non-collagenous protein, reported that about half of the [^3H]-proline and [^3H]-glycine in the periodontal ligament of mouse molars is incorporated into collagen. Their material, however, was fixed in Carnoy's fluid and demineralized in a mixture of formic acid and sodium citrate. As our study suggests that, as a result of this treatment collagen may be lost from the ligament, the true ratio between the label incorporated in collagen and non-collagenous protein might be different from that given by Carneiro and Leblond.

The use of quantitative autoradiographic techniques can give valuable information about various aspects of protein metabolism in the periodontal ligament. The quantity of the label, however, can be influenced by the histological procedure chosen. To minimize loss of radioactivity during tissue processing, Bouin's fixative seems to be a more adequate fixative for the periodontal ligament than formalin or Carnoy's fluid, whereas EDTA as a demineralizing agent might be preferable to a mixture of formic acid and sodium citrate particularly when Carnoy's fluid is used for fixation.

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