AN ARTEFACT IN RADIOAUTOGRAPHY DUE TO BINDING OF FREE AMINO ACIDS TO TISSUES BY FIXATIVES

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ABSTRACT

The binding of labeled free amino acids to liver and to purified protein by commonly used fixatives was investigated. Glutaraldehyde caused 25% of free leucine to be bound to serum albumin in solution, whereas formaldehyde bound only 0.5%. Liver slices were incubated for 2 min in the presence of labeled leucine and of puromycin, which permits absorption of leucine into the cell but inhibits incorporation into protein. Both counting and radioautographic techniques showed that glutaraldehyde bound 30 times, and osmic acid six times, as much free amino acid as did formaldehyde. By comparing liver slices incubated with and without puromycin for 2 min, it was calculated that in radioautographs prepared after fixation with glutaraldehyde, osmic acid, or formaldehyde 63, 25, and 4% respectively of the grains were due to binding of free amino acid. Formaldehyde, freshly prepared from paraformaldehyde, gives good preservation and is the recommended fixative for radioautography. When levels of free substrate in a tissue are high at the time fixative is added, the amount of binding of free substrate induced by the fixative should be included as a control in radioautographic experiments.

INTRODUCTION

Radioautography is a useful technique for determining the intracellular location of radioactive compounds. When the synthesis of biological macromolecules, such as proteins, nucleic acids, and polysaccharides, is studied by electron microscopy, the tissues usually are fixed with osmium tetroxide or glutaraldehyde followed by osmium tetroxide prior to dehydration, embedding, and sectioning.

Users of radioautography customarily have determined the number of grains, or background, present in the emulsion itself or induced during its application, storage, and development. The possibility has been discounted largely that artefacts may be caused by retention of the radioactive substrate molecules as well as the desired molecules. Droz and Warshawsky (1) have studied the use of Bouin's fixative for light microscopic radioautography of tissues 30 min and 24 hr following in vivo administration of labeled leucine. Under their experimental conditions the tissues contained very little free amino acid at the time the fixative was added, and, therefore, binding attributable to the fixative was insignificant. A quantitative estimate of artefacts induced by the fixative is especially important, however, in experiments in which a significant amount of the added radioactive material is still present as a free compound. This condition is encountered when the exposure time is short or when the amount of substrate administered is relatively large.

The present study is a quantitative comparison

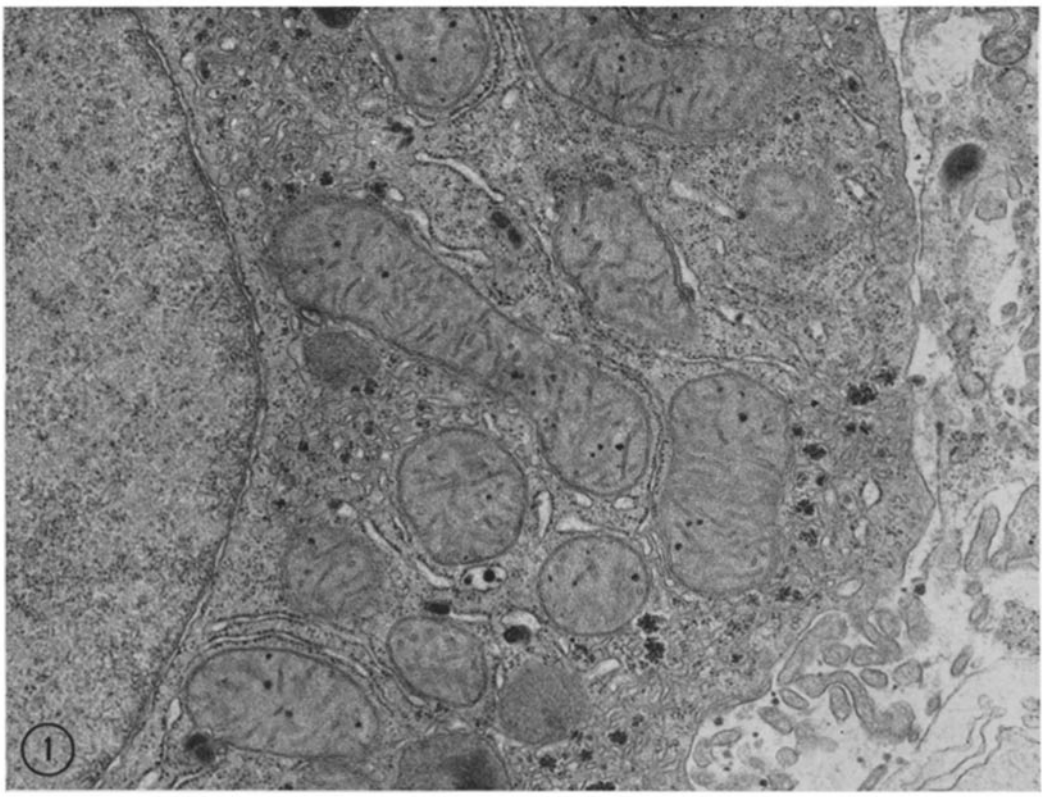

FIGURE 1 Portion of rat liver cell showing cytologic detail after formaldehyde fixation. Postfixed in osmium tetroxide and stained by uranyl acetate and lead citrate. × 20,000.

of the binding of labeled amino acid to tissue structures induced by several fixatives commonly used in electron microscopy. The inhibitor compound, puromycin, was employed to block synthesis of protein without blocking the uptake of free amino acids by cells. Glutaraldehyde was found to bind significant quantities of free amino acids to tissues, and even osmium tetroxide alone caused appreciable binding. This bound amino acid is not removed during subsequent washing and dehydration. Use of freshly prepared formaldehyde as fixative is recommended.

MATERIALS AND METHODS

Reagents

The glutaraldehyde used was a 3% solution prepared from 25% glutaraldehyde (Union Carbide Corporation, New York) in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.005% $CaCl_2$. The osmium tetroxide was a 2% solution in the same buffer. 4% formaldehyde was prepared freshly by suspending 4 g of paraformaldehyde (No. 421, Eastman Chemical Products, Inc., Kingsport, Tenn.) in 80 ml of water containing the appropriate amount of salt for 100 ml of the pH 7.3 phosphate buffer. The suspension was heated to 70°C with stirring, cooled, filtered, and the volume adjusted to 100 ml. The calcium chloride was added after filtering. The incubation medium was Krebs-Ringer-bicarbonate (Na = 144 mM, K = 6 mM, Ca = 2.4 mM, Mg = 1.2 mM, Cl = 117 mM, HCO_3 = 35 mM, PO_4 = 1.4 mM, SO_4 = 1.2 mM, pH 7.4) containing 0.02% glucose and 0.0004% phenol red, prepared daily from stock solutions and equilibrated with 5% CO_2–95% O_2. When desired, solid puromycin dihydrochloride (Nutritional Biochemicals Corp., Cleveland, O.) plus an equivalent amount of 1 N $NaHCO_3$ was added to yield 2 mM puromycin. When leucine-^{3}H was employed, 75 μc of ^{3}H-4,5-L-leucine, 7.6

c/mmole (TRK-170, Nuclear-Chicago Corporation, Des Plaines, Ill.), was lyophilized in the incubation vial and 0.10 ml of medium added just before use. When leucine-^{14}C was used, 1 μc of U-^{14}C-L-leucine, 6.8 mc/mmole (Calbiochem, Los Angeles, Calif.), in 0.02 ml of water was added to 1 ml of medium. Both of the isotopic preparations were tested by paper chromatography and radioautography and shown to be over 99% pure within 1 month prior to their use.

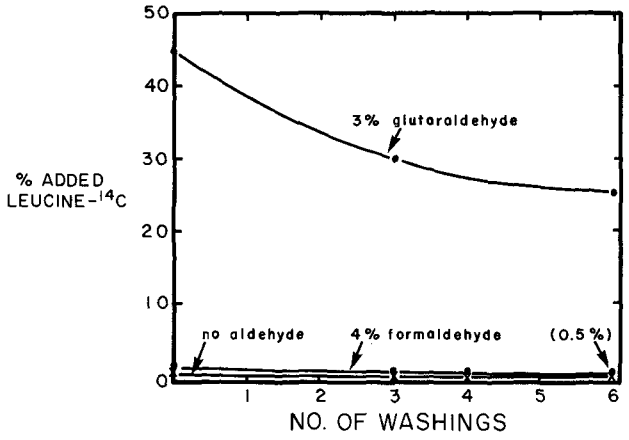

FIGURE 2 Binding of free leucine to bovine serum albumin by aldehyde fixatives. The abscissa shows the number of washings with 5% trichloroacetic acid. The ordinate shows the percent of added leucine-^{14}C bound by albumin.

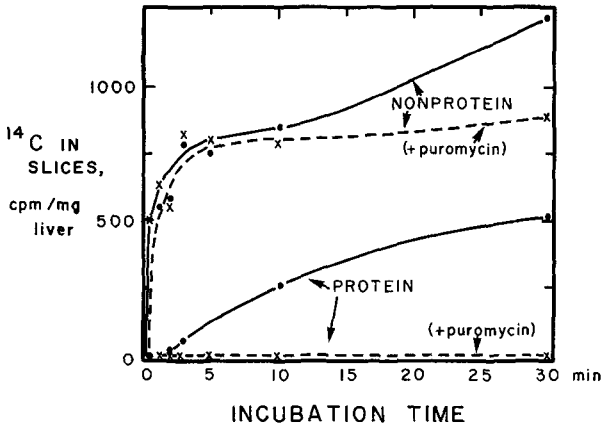

FIGURE 3 Uptake of labeled leucine by rat liver slices on incubation at 38°C. The upper two curves are the trichloroacetic acid-soluble counts and the lower two curves are the trichloroacetic acid-insoluble counts.

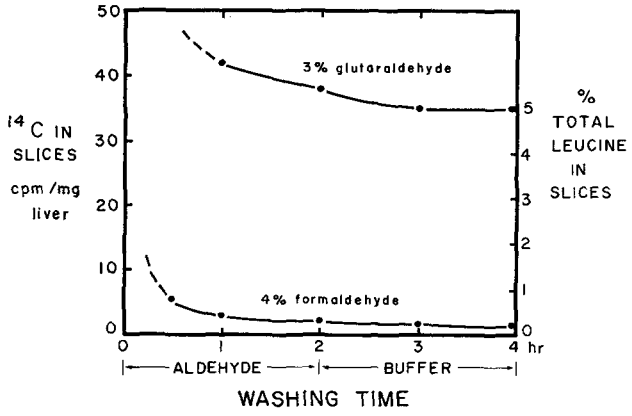

FIGURE 4 Retention of free leucine in liver slices after treatment with aldehyde fixatives. The liver slices had been incubated for 2 min in leucine-^{14}C to which 2 mM puromycin was added to block synthesis of proteins.

TABLE I

Calculation of Fixative-Bound Leucine as % of Total Bound Leucine after a 2 min Incubation Period

Figures are counts per minute per milligram (cpm/mg) wet liver. The total free leucine present in the slices before fixation was 680 cpm/mg. Protein-bound leucine was determined as that precipitated by trichloracetic acid. Fixative-bound leucine was taken to be that found when the slices were incubated in the presence of puromycin.

	Fixative		
	Glutaraldehyde	Osmium tetroxide	Formaldehyde
Protein-bound leucine	20	20	20
Fixative-bound leucine	34	6.8	0.9
Total bound leucine	54	26.8	20.9
Fixative-bound leucine / Total bound leucine	63%	25%	4%

Liver Slice Incubations and Counting

Male Sprague-Dawley rats weighing 100–250 g were fasted for 18–24 hr, and sacrificed by a blow on the head; the right lobe of the liver was chilled quickly in incubation medium at 0°C. Slices 0.3–0.5 mm thick were cut with a Stadie-Riggs tissue slicer in the same medium at 25°C.

For experiments in which the binding of amino acid was detected in a flow counter, leucine-^{14}C was employed. Slices were rinsed with shaking for 30 min in medium with or without puromycin at 38°C in an Erlenmeyer flask flushed with 5% CO_2-95% O_2, then placed for 2 min in 5 volumes of medium which contained leucine-^{14}C. In some instances the radioactive leucine was "chased" at the end of 2 min by adding a large volume of warm medium which contains 5 mM L-leucine (Nutritional Biochemicals Corp.) which was changed several times as the incubation was continued. At the end of the incubation, some of the slices were processed in fixatives as described below. Specimens in glutaraldehyde or formaldehyde were taken only through the aldehyde and buffer washing steps, then plated on 12.5 mm platinum discs with 88% formic acid for counting. Specimens in osmium tetroxide were processed through propylene oxide, then ground and plated after heating with an equal weight of NaOH in concentrated solution. Correction was made for the weight of the NaOH in calculation of counts. Other slices were blotted and ground in 5% trichloroacetic acid at 0°C. These were washed four times in 20 volumes of 5% trichloroacetic acid, heated to 90°C for 15 min in the same solution, and washed once more at 0°C. They then were washed three times in 1/1 ethanol/ether and once in ether; samples of about 5 mg were plated with formic acid on 12.5 mm platinum discs. ^{14}C was determined in a Picker thin-window gas flow counter with on efficiency of about 35% for ^{14}C and a background of 6–8 cpm. Counts were corrected to 100% efficiency and 0 thickness, using previously determined factors which ranged from 0.15 to 0.35 depending on the dry weight of the sample.

For radioautography with leucine-^{3}H, tissue slices 0.3–0.5 mm thick were cut into 3 × 4 mm rectangles with fine scissors, and rinsed for 30 min at 38°C in medium containing 2 mM puromycin in an Erlenmeyer flask. Individual slices were placed in flushed 11 × 40 mm vials in 0.10 ml of medium plus puromycin plus leucine-^{3}H at 38°C. After 2 min the slices were rinsed briefly in 5 ml of medium and the fixative was added.

Tissue Preparation for Electron Microscopy

Blocks of 1 mm^3 or incubated slices were fixed at room temperature for 2 hr in 3% glutaraldehyde or 4% formaldehyde with agitation. The fixative was changed 10 times during the 2 hr. Tissues then were washed (four changes) for 2 hr in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.005% $CaCl_2$ and 10% sucrose. Together with the specimens fixed in osmium tetroxide alone, they then were transferred to osmium tetroxide for 2 hr,

TABLE II

Estimated Percentage of Fixative-Bound Leucine Anticipated Under Various Conditions of Incubation

Incubation time	% of grains due to fixative-bound leucine		
	Glutaraldehyde	Osmium tetroxide	Formaldehyde
min			
1	86	54	13
2	63	25	4
5	36	10	1
2 + 3 min chase	16	4	0
2 + 15 min chase	3	1	0

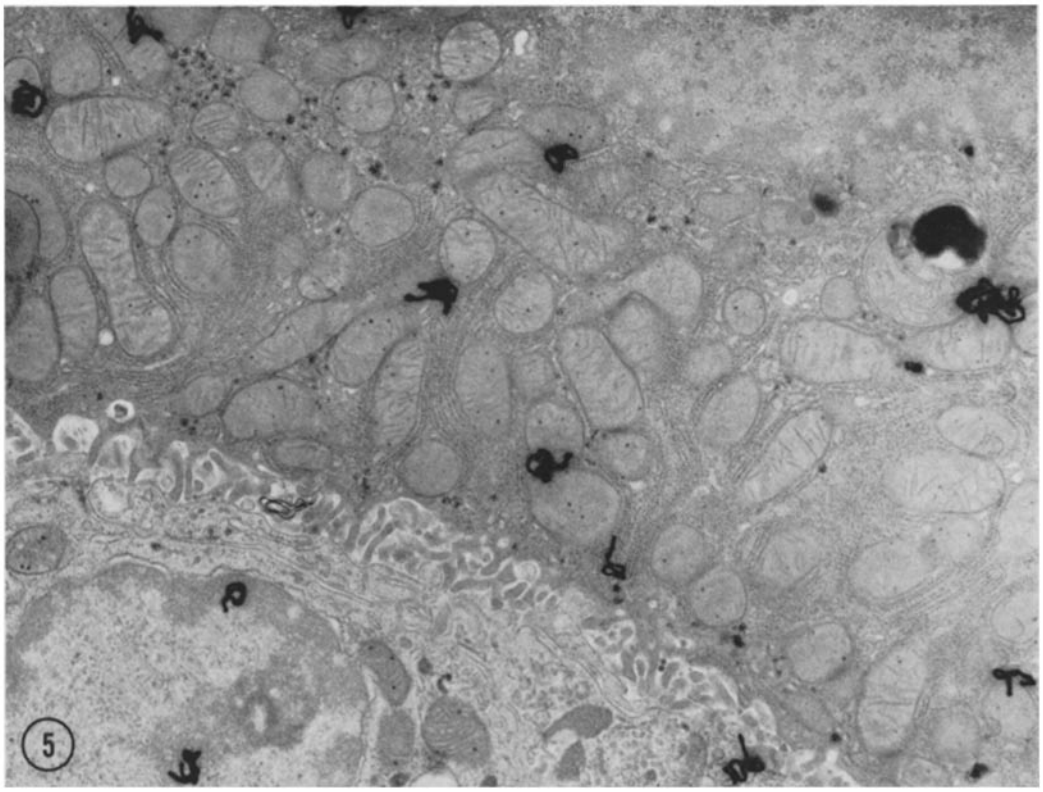

FIGURE 5 Radioautograph of rat lives slice fixed in glutaraldehyde after 2 min incubation in presence of leucine-^{3}H and puromycin. 12 grains are shown representing retention of free amino acid. Postfixed in osmium tetroxide; uranyl acetate and lead citrate stain. × 12,800.

rinsed briefly in water, dehydrated in a graded series of ethanol solutions (50, 70, 95, 100%) at 30 min intervals, and embedded in 1/1 Epon mixture by the usual technique. Slices were oriented vertically in gelatin capsules. Sections cut with a diamond knife were stained with uranyl acetate and lead citrate and examined in an RCA-3G electron microscope operated at 100 kv using a 200 μ condenser with a 35 μ objective aperture.

For radioautography, "pale gold" sections were placed on collodion-covered slides. They were stained and coated with Ilford L-4 emulsion according to methods of Salpeter and Bachmann (3). Slides were stored at 4°C in boxes containing Drierite (W. A. Hammond Drierite Company, Xenia, O.) for 10 days, developed in Microdol-× (Eastman Kodak Co. Rochester, N.Y.) for 3 min following gold latensification. After an acid stop solution, a fixative containing 200 g of sodium thiosulfate and 20.5 g of sodium metabisulfite per liter was used.

RESULTS

Cytology with Formaldehyde Fixation

Formaldehyde solutions freshly prepared from paraformaldehyde under conditions similar to those described by Pease (2) do not contain the methyl alcohol preservative and the formic acid present even in reagent grade formaldehyde solutions. The preservation of cytologic detail of liver blocks and slices fixed in fresh formaldehyde and postfixed in osmium tetroxide was comparable to that obtained with the combination of glutaraldehyde and osmium tetroxide (Fig. 1).

Binding of Leucine to Albumin by Aldehyde Fixation

Glutaraldehyde or formaldehyde was added to a 12% solution of serum albumin in 0.15 M NaCl

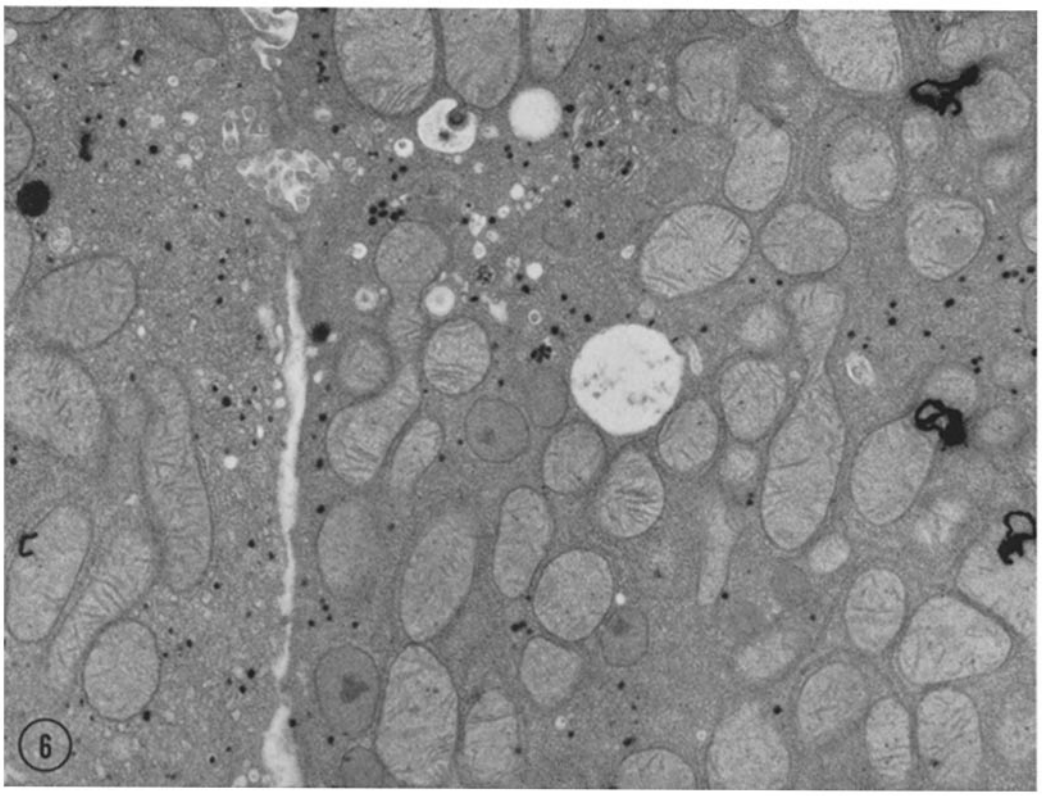

FIGURE 6 Radioautograph prepared as for Fig. 5 except osmium tetroxide fixation. Four grains are shown. × 12,800.

containing 0.15 mM leucine-^{14}C at 23°C. After 15 min the protein was precipitated with 5% trichloracetic acid at 0°C and the precipitates were washed repeatedly. The counts remaining in the precipitate were determined and the results are shown in Fig. 2. Following glutaraldehyde treatment some leucine was removed by repeated washing; however, more than 25% of the added leucine remained with the precipitated protein after six washings. Following formaldehyde treatment, only 0.5% of the added leucine remained with the precipitated protein, approaching the amount present in the trichloracetic acid control.

Binding of Leucine to Liver Slices by Fixative: Counting Studies

The kinetics of incorporation of labeled leucine into liver slices are shown in Fig. 3. The two upper curves, representing trichloracetic acid–soluble counts, show the rapid absorption into the slices with or without puromycin. The middle line shows incorporation into protein, measured as the amount of radioactivity in the precipitate. The bottom line shows the minimal incorporation into protein when the slices were incubated with puromycin, despite uptake into the slices comparable to that obtained when slices were incubated without puromycin. An incubation of 2 min in the presence of puromycin was selected to assess the binding of free leucine by the fixatives, since at this time near-maximal uptake of the leucine occurred but little leucine was incorporated into protein even in the absence of puromycin. The slices were washed repeatedly in both the fixative and washing buffer. After 2 hr of washing, liver slices fixed in formaldehyde contained very little leucine, whereas liver slices fixed in glutaraldehyde contained 5% of the total leucine absorbed into the slices (Fig. 4).

Values obtained on the percentage of free amino acid bound to liver slices by osmium tetroxide, glutaraldehyde, and formaldehyde were com-

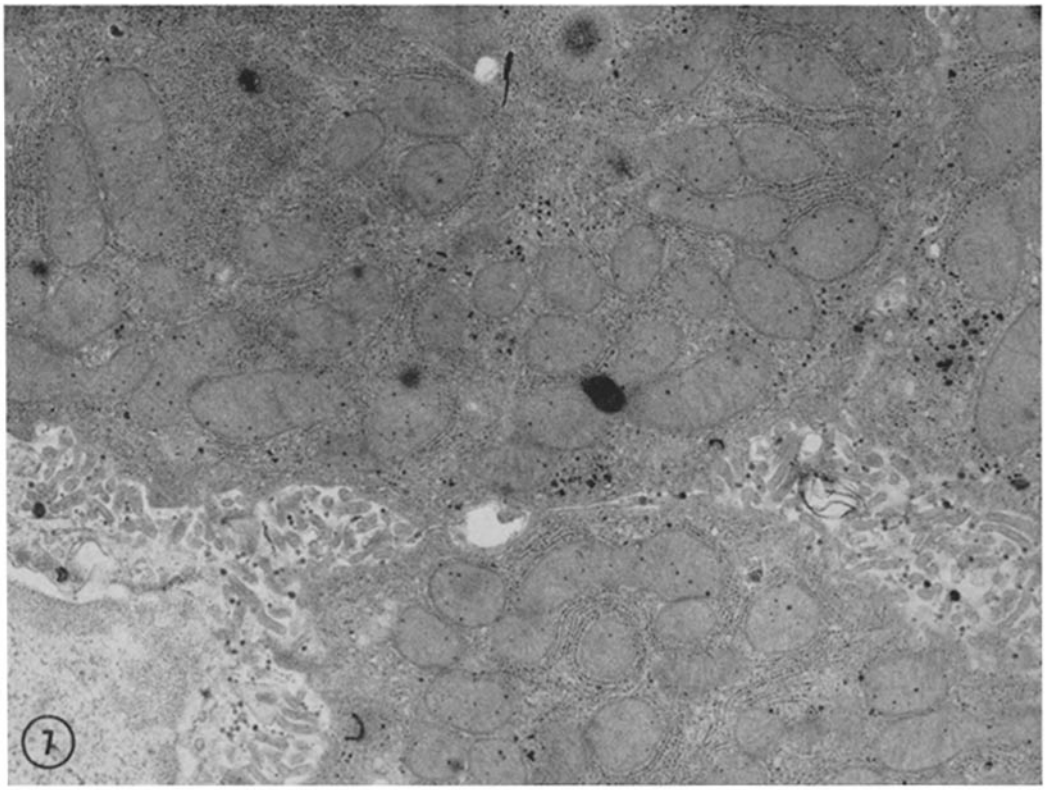

FIGURE 7 Radioautograph prepared as for Fig. 5 except formaldehyde fixation, postfixed in osmium tetroxide acid. One grain is shown. × 12,800.

bined with data from the studies of incorporation of leucine into proteins of the liver. Table I shows these calculations for a 2 min incubation. The slices contained 680 cpm per mg of liver due to free amino acid present and 20 cpm due to the amino acid synthesized into protein. Addition of fixative caused the binding by tissue of radioactive leucine measured by 34 cpm for glutaraldehyde, 6.8 for osmium tetroxide, and 0.9 for formaldehyde. In radioautographs prepared from these materials, 63% of the grains in the glutaraldehyde-fixed tissues could be attributed to the binding of free amino acids, 25% in the osmium tetroxide-fixed tissues, and only 4% in formaldehyde-fixed tissues.

Similar calculations were made for slices incubated for various times followed by further incubation in nonradioactive medium (Table II). Under all conditions of fixation with osmium tetroxide or glutaraldehyde, except with a long incubation in a nonradioactive "chase," a significant proportion of the counts was due to binding of free amino acid. This was not true for formaldehyde. These figures are based on a specific rate of protein synthesis and might vary among experiments, but the comparison for the different fixatives would remain the same.

Binding of Leucine to Liver Slices by Fixative: Radioautographic Studies

Liver slices were incubated for 2 min in medium which contained puromycin and leucine-^{3}H, fixed in each of the three fixatives, and radioautographs were prepared. After 10 days of exposure the slides were developed, electron micrographs were taken, and the grains in an area of approximately 2000 μ^2 were counted. Liver fixed in glutaraldehyde had 99 gr/1000 μ^2, that fixed in osmium tetroxide had 19 gr/1000 μ^2, whereas formaldehyde-fixed liver had 3.3 gr/1000 μ^2. Expressed as grains per $3\frac{1}{4} \times 4$ inches plate taken at a magnification of 4,500, the fixatives resulted in the following num-

ber of grains per plate: glutaraldehyde, 33; osmium tetroxide, 6.3; formaldehyde, 1.1. In Figs. 5, 6, and 7 are shown representative radioautographs of equal areas prepared from liver fixed in the three fixatives.

DISCUSSION

Artefacts induced by the method of tissue fixation in radioautographic studies of synthesis of proteins, nucleic acids, and other large molecules have received little attention. In this investigation both glutaraldehyde and osmium tetroxide were shown to bind significant amounts of free amino acid to tissue structures, although freshly prepared formaldehyde bound little. Glutaraldehyde is an excellent fixative for cytologic detail, probably because its two aldehyde groups can cross-link large molecules. However, apparently it also links small molecules by means of their amino groups to tissue proteins and nucleic acids and thus may be a source of serious artefacts in radioautographic studies.

Formaldehyde, when freshly prepared from paraformaldehyde, gave good preservation of cytologic detail and seems the fixative of choice for radioautography.

Regardless of the fixative used, the amount of binding produced by the fixative under the conditions of a specific experiment should be determined. This can be done by adding inhibitors of incorporation, such as puromycin or actinomycin, to tissues which are then processed in the same manner as the experimental tissues. With these controls it is possible to know the proportion of grains located over free substrate molecules or their breakdown products, compared to those located over macromolecules into which the substrate has become incorporated.

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