AUTORADIOGRAPHIC DETERMINATION OF S³⁵ IN TISSUES AFTER INJECTION OF METHIONINE-S³⁵ AND SODIUM SULFATE-S³⁵*

By LEON C. EDWARDS,‡ M.D., and K. N. UDUPA, F.R.C.S. (C.)§

(*From the Department of Surgery, Harvard Medical School, and The Fifth Surgical Service and Sears Surgical Laboratory, Boston City Hospital*)

PLATES 243 TO 245

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Sulfur-35 disintegrates with the release of low energy or "soft" beta particles which are capable of producing autoradiograms of excellent resolution. The quality of the autoradiograms, and consequently their usefulness as localizing indicators of the isotope in tissue, depends upon the over-all technic of processing the tissue samples as well as upon the dose and form of the S³⁵. The work reported below deals with a number of technical problems encountered in the use of S³⁵ in studies of the sulfur metabolism of healing wounds.

Following intraperitoneal injection into rats as either radiomethionine or radiosulfate, the isotope is rapidly distributed throughout the body, and a substantial part of the injected dose is excreted during the ensuing 24 to 48 hours (1, 2). The fraction of isotope bound by regenerating connective tissue after methionine injection is largely incorporated into proteins (3), and to a less extent, after oxidation of the amino acid sulfur, into the sulfated mucopolysaccharides of the ground substance (4, 5). The tissue-bound fraction of the isotope injected as sodium sulfate is incorporated almost exclusively into the sulfated mucopolysaccharides of the wound (4, 5).

The large protein and polysaccharide molecules into which sulfur is incorporated are so much less soluble than the free sulfate ion that these substances are usually regarded as insoluble in solvents used in their preparation for autoradiographic localization. A common procedure, for instance, to rid the tissue

757

of sulfate ion and retain bound sulfur for localization employs aqueous fixatives followed by prolonged washing (6). Quantitative evidence supports the assumptions about the insolubility of bound sulfur on which this procedure is based. Kodicek (5) has shown that the radiosulfur content of excised granulation tissue gained by incubation in $Na_2S^{35}O_4$ is not appreciably reduced by washing in Ringer's solution for a period of 30 hours. This suggests that the intact tissue specimen suffers no appreciable loss of bound sulfate in an aqueous solution. Our work suggests that the same is true of protein-bound S^{35} after the injection of radio-methionine.

It is surprising, therefore, that autoradiograms of tissue obtained 48 hours or more after injection of $Na_2S^{35}O_4$ or methionine-S^{35}, and fixed in a variety of aqueous and organic solvents, show a consistent and striking loss of the isotope when compared with parallel autoradiograms of frozen-dried tissues cut at half the thickness. The work reported below was undertaken to discover the particular steps in the usual histological procedure at which the bound isotope is lost. The experimental approach is based upon the assumption that nearly all of the S^{35} present in the tissues 48 hours or more after injection is bound into proteins and polysaccharides.

Methods

In order to relate isotope loss to the method of preservation and to the technic of autoradiography employed, parallel autoradiograms were made by each of three methods, which are referred to hereafter as *coated, floated*, and *dry-mounted* autoradiograms. One group of four young adult rats received methionine-S^{35} and the other $Na_2S^{35}O_4$ intraperitoneally in a dose of 70 μc. S^{35}/100 gm. body weight 3 days after the infliction of standardized surface wounds. Granulation tissue was harvested 12, 24, 48, and 100 hours after the injection. A part of each specimen was digested and its S^{35} content measured with a Robinson flow counter. The re-remainder of the specimen was divided into several parts for histological work. The aqueous fixatives selected for comparison were formalin, saturated lead subacetate, lead acetate, Bouin's and Zenker's solutions; the only two organic solvent mixtures used were methanol-ethanol and Carnoy's solution. Comparable tissue samples for dry mounting were preserved by quenching in liquid nitrogen and freeze-drying. All tissues were infiltrated and embedded for cutting in tissue mat. Sections were prepared for autoradiographic comparison in the ways described below. All plates were prepared with Eastman NTB-3 emulsion, diluted to obtain a film thickness of approximately 3 microns. Exposure and photographic processing were identical for all preparations.

Coated autoradiograms were prepared according to the method of Gross *et al.* (7). Six micron sections were mounted on glass slides from a water floatation bath, cleared, hydrated, and stained prior to coating the sections with the nuclear track emulsion. The plates were allowed to dry at room temperature and exposed in sealed, light-tight plastic boxes at 4°C. Sections mounted by this method were subjected to extensive solvent contact prior to autoradiography.

Floated autoradiograms were prepared with 6 micron sections which were cut and stored as dry paraffin ribbons. They were then subjected to the brief contact with water entailed by floating the ribbons onto previously prepared nuclear plates in the dark room.

Dry-mounted autoradiograms of frozen-dried tissue were prepared by an adhesive-transfer method (8), in which a short strip of adhesive-transfer cellophane tape is applied to the face of the block to provide backing for the individual 3 micron sections during cutting and handling.

In the dark room the sections were firmly sealed by means of the tape backing directly to the slightly tacky emulsion surface of nuclear plates freshly coated with emulsion. A coating device for this purpose has been described elsewhere (9). The autoradiograms were developed after exposures ranging up to 20 weeks. Photographic processing of all plates was carried out in Eastman D-19 developer at 20° C. for 1 minute, followed by a cold water rinse; 5 minutes in 30 per cent hypo, and a final 30 minute wash in cold running water. This was followed by routine staining and covering.

Results

Representative curves of the content of S^{35} in granulation tissue at intervals up to 100 hours after injection of the isotope as either methionine-S^{35}
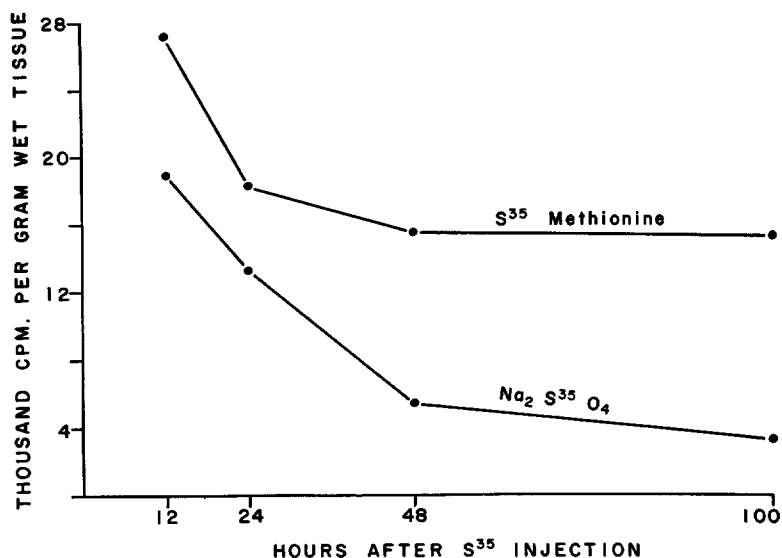
TEXT-FIG. 1. Content of S^{35} in granulation tissue after intraperitoneal injection of 70 μc./100 gm. weight in wounded rats.

or $Na_2S^{35}O_4$ are plotted in Text-fig. 1. On the basis of the work already cited, the high but rapidly falling early activity shown is largely produced by the isotope in an unbound form, a large fraction of which is excreted during the first 48 hours. A comparison of autoradiograms prepared by dry-mounting frozen dried tissues obtained 12 and 24 hours after injection with autoradiograms of the same tissues after fluid fixation and wet mounting, reveals the greatest loss of the isotope from the latter during this period. The loss is more marked in the tissues of animals injected with $Na_2S^{35}O_4$. Autoradiographic observations, coupled with the earlier and more complete flattening of the curve representing S^{35} content of granulation tissue after the injection of methionine-S^{35}, suggest earlier and more complete binding of the isotope after its injection in

the form of the amino acid. However, autoradiograms prepared from tissues obtained 48 hours or more after injection of the isotope and compared to detect losses of bound S^{35} reveal no consistent differences between animals which received methionine-S^{35} and those which received Na$_2$S^{35}O$_4$.

Fig. 1 illustrates the relative density of autoradiograms of tissue from the same animal 100 hours after injection of methionine-S^{35}. They illustrate the results obtained respectively in *coated*, *floated*, and *dry-mounted* preparations. The lowest concentration of silver granules is seen in the *coated* autoradiogram (*A*), and the highest density in the *dry-mounted* preparation (*C*). The *floated* autoradiogram (*B*) reveals a density of silver granules between these two. It can be concluded that accidental loss of the isotope from the sections is greatest in the coated series which suffered the greatest exposure to solvents and least in the *dry-mounted* preparation which received no solvent contact except that presented by molten paraffin.

The more pronounced density of nuclear staining in the *dry-mounted* autoradiogram (Fig. 1 *C*) must be attributed to the method of preservation used, since staining and photographic processing were identical in the three series. The autoradiographic reaction of *coated* and *floated* preparations is so weak that it is frequently indistinguishable from background fogging. None of the dry-mounted autoradiograms present this problem. The possibility that the marked and consistently greater density of silver granules in the *dry-mounted* plates is an artifact peculiar to this method of tissue preservation and mounting is ruled out by previous experience with the technic (15).

Fluid-fixed tissues mounted by a method involving solvent contact generally reveal a substantial loss of the isotope. The loss is comparatively less after fixation by saturated lead subacetate or by methanol-ethanol.

Losses of the isotope (introduced as methionine-S^{35}) ascribable to solvent exposure during mounting were found in the skin, skin appendages, muscle, and mature fascia adjacent to the wound as well as in liver and other tissues. The loss of presumably bound sulfur illustrated in Figs. 1 *A* to *C* is, therefore, not confined to regenerating connective tissue. A similar loss of bound isotope is seen in *coated* and *floated* autoradiograms after its injection as Na$_2$S^{35}O$_4$. It appears, therefore, that whether by dissolution or by mechanical erosion, the ribbon or section which is exposed to solvents loses the isotope whereas the intact tissue specimen, although bathed for prolonged periods by solvents during fixation, does not.

An explanation for the apparently contradictory behavior of bound S^{35} in the intact specimen during prolonged exposure to fluid fixatives, and in the tissue section exposed for a much shorter time to a variety of solvents in histological processing, is suggested by the photomicrographs in Fig. 2. Both of the *floated* autoradiograms shown in Fig. 2 suffered drastic loss of the isotope as compared with *dry-mounted* autoradiograms of the same tissue

prepared by freeze-drying. In addition to this, a complete loss of the isotope from the marginal tissue of both sections is seen. These autoradiograms illustrate how solvent action and fixation proceed simultaneously in the system (10) until fixation has proceeded to such an extent that the bound isotope is largely protected from further dissolution. It suggests as well that the marginal tissue of the intact tissue sample provides a physical barrier to the loss of the macro-molecular sulfur-containing substances, whether this takes place by diffusion in solution, or by particulate erosion. Similar marginal loss of the bound isotope is not apparent at the skin surface (a thin natural barrier) but is found to a varied extent at the cut edges of the mature subcutaneous tissues, as well as in granulation tissue which is shown in Fig. 2. No peripheral loss of isotope is seen in *dry-mounted* autoradiograms of frozen dried tissue.

Discussion

On the basis of the observations reported here, the common assumption that protein- and polysaccharide-bound sulfur is not subject to appreciable accidental loss during preparation of the tissue for autoradiography is in error. Even when the radioactive content is sufficient to give an autoradiogram of useful density, the tissue section which is in contact with solvents during mounting suffers a serious loss of isotope as compared with dry mounted sections of the same tissue. In the presence of partial, but not prohibitive loss of isotope, displacement of the isotope within the tissue is a potential hazard, although an unproved one.

The severe loss of isotope revealed by *floated* autoradiograms is surprising in view of the brief contact between the paraffin ribbon and cold water during mounting. A similar loss of P^{32} from paraffin ribbons of tissue subjected to brief floatation on water has been reported by Holt *et al.* (11). These authors attributed the loss entirely to the fraction of injected P^{32} not bound in nucleic acids and other relatively insoluble tissue constituents. The loss of presumably protein- and polysaccharide-bound sulfur described above suggests the possibility of a partial loss of bound phosphorus, as well as the unbound ion, in the work cited.

The superiority of methanol-ethanol and lead subacetate fixation over the other fixatives used in preventing subsequent loss of bound S^{35} may be related to a similar superiority of metachromatic staining seen in growing connective tissue fixed in these solvents. Metachromatic staining is thought by Walton (12) and others (13) to depend largely upon the highly ionized sulfate ester of the mucopolysaccharides of the ground substance. The more complete precipitation of these substances by lead subacetate, which results in less subsequent loss of the isotope during preparation for autoradiography, probably depends upon the distinctive properties cited by Cohn in comparing lead subacetate with other heavy metal plasma protein precipitants. "Lead sub-

acetate is a general precipitant. In addition to rendering insoluble the acid glycoprotein, it precipitates non-protein components of plasma, including the blood group specific polysaccharides and peptides" (14). The general class of protein and polysaccharide compounds cited by Cohn contain most of the bound S^{35} after injection as sulfate.

The marginal loss of isotope observed in tissues fixed in fluids (alcohols or aqueous fixative mixture) is attributed to solvent action during fixation because it occurs in addition to the generalized loss of isotope resulting from floatation of the ribbon. This observation, coupled with the absence of appreciable isotope loss on prolonged washing of the intact specimen, suggests that the marginal tissue presents a diffusion barrier between the central tissue mass and the solvent. The notion that the marginal tissue protects the central mass during fixation is important because it suggests the possibility that the isotope may be displaced within large microscopic regions of the tissue, although prohibitive loss occurs only at the margins.

Many of the *coated* and *floated* autoradiograms made during this experiment were ruined by chemical fogging during exposure. No steps were taken to assure dryness of the plates other than sealing the dried plates in light-tight plastic boxes. Diffuse fogging of the *dry-mounted* autoradiograms sometimes occurred outside the area covered by the adhesive-transfer tape. Little or no fogging of the emulsion was seen in the region of the section itself which is protected by the tape.

The discrimination of chemical fogging from the true detection of radioactivity by nuclear emulsion is not a problem when dense and discrete tracks, such as those produced by alpha particles and protons, are formed. It is seldom a problem with low energy beta particle autoradiograms (C^{14}, S^{35}) of the density shown in Figs. 1 C and 3. However, underexposed and overexposed autoradiograms of soft beta particles, as well as those of high energy beta particles (P^{32}, I^{131}), present microscopically diffuse granulation which is more easily confused with accidental fogging. Since fogging from stray radiation is easily avoided, except in autoradiography involving irradiation of the nuclear track plate (15), only two varieties of chemical fogging are ordinarily encountered. In one form there is diffuse fogging of the entire emulsion which characteristically occurs in contaminated or improperly dried and stored plates. This artifact is recognized because it is independent of the tissue section and the silver grains are frequently aggregated in coarse, irregular clumps, quite unlike anything produced by radioactivity alone. The other common form of fogging is due to chemical action of tissue components in direct contact with moist emulsion. A tissue "chemogram" of considerable beauty, but questionable utility is frequently produced. The "chemogram" is recognized by (1) its striking uniformity of granule size (0.2 to 0.3 microns, NTB-3 emulsion) and smoothness of distribution with no tendency towards the minute aggregation or track formation seen in Fig. 1 C; (2) its failure to show localized granules at the site

of known concentration of the isotope used, such as the hair follicle for S^{35}; and (3) its diffusely smooth border at or near the edge of the section or tissue structure responsible for the chemical action in contrast to the minutely irregular pattern of diminishing density seen at the same sites in the autoradiogram. By these criteria the granulation shown in Fig. 1 A cannot be identified with certainty as chemical artifact or the true detection of radioactivity. This serves, however, to strengthen the contrast between the results obtained by the methods illustrated in Figs. 1 A and 1 C. The common tendency to over-expose autoradiograms reduces the value of the discriminating features described above, as well as the value of the autoradiogram by obscuring the associated tissue components. Fig. 3 illustrates an ideally exposed autoradiogram of cartilage S^{35} in the rat.

SUMMARY

The loss of "bound" S^{35} that occurs during various mounting procedures used in autoradiography was studied in healing surface wounds of rats treated with either methionine-S^{35} or $Na_2S^{35}O_4$. Valid autoradiography of bound S^{35} in this tissue is not possible until 48 hours after radiosulfate and 24 hours after radiomethionine injection, when the S^{35} is almost entirely bound in large protein and polysaccharide molecules.

Autoradiograms of S^{35} given in both the organic and inorganic form reveal substantial over-all loss of the bound isotope from sections subjected to contact with solvents prior to autoradiography. A comparison of autoradiograms prepared by *dry-mounting* sections of frozen-dried tissue with autoradiograms of wet-mounted sections of the same tissue suggest that the loss is proportional to the extent of the contact with solvents. Evidence suggests that loss of the isotope occurs during contact of the ribbon or section itself with solutions after fixation and cutting and prior to radiation exposure. No appreciable loss of the bound isotope seems to occur during contact of the intact tissue specimen with a variety of fluid fixatives except for a marginal zone at the excision edges of the tissue. The potential hazard of displacement of the isotope during fixation, however, remains.

Technics which prevent loss of the isotope and fogging of the nuclear emulsion permit the use of thinner sections and emulsion films and the fine resolution of image rendered possible by the physical properties of S^{35}.

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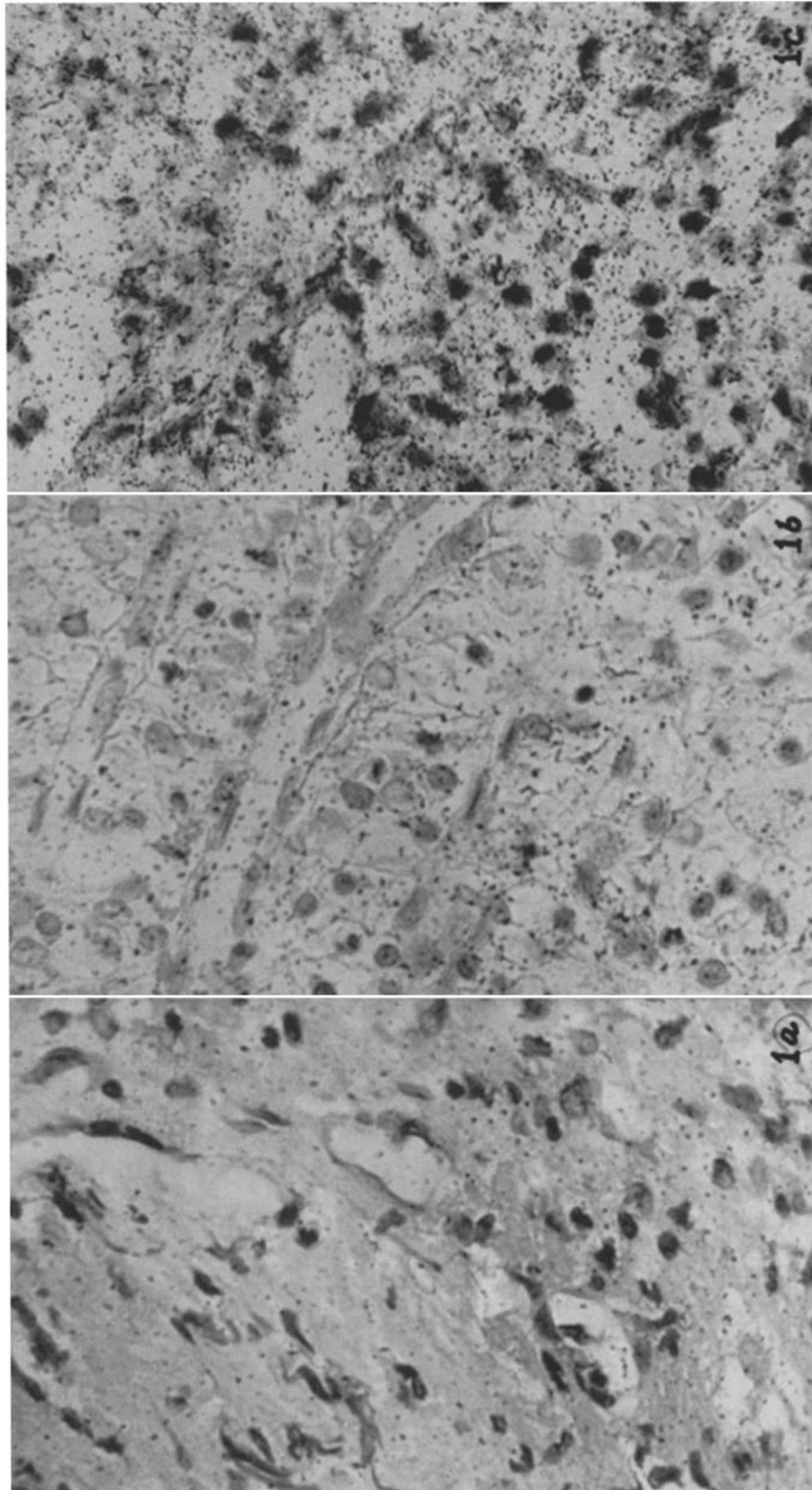
EXPLANATION OF PLATES

PLATE 243

FIG. 1. Autoradiograms of S^{35} in samples of tissue collected from the same wound 100 hours after injection of methionine-S^{35} but fixed and mounted by different procedures.

FIG. 1 *A*. 6 micron section cleared and stained with hematoxylin and eosin prior to coating with nuclear emulsion. Lead acetate fixative. $\times$ 1000.

FIG. 1 *B*. 6 micron section mounted on the nuclear plate by brief floatation in water. Lead acetate fixative.

FIG. 1 *C*. 3 micron section of tissue prepared by freeze-drying, and cut and mounted by a dry, adhesive-transfer method. Notice that this section, which shows the most evidence of radioactivity, is cut at half the thickness of the other two.

FIGS. 1 *B* and *C* stained with hematoxylin and dilute eosin after autoradiography. $\times$ 1000.
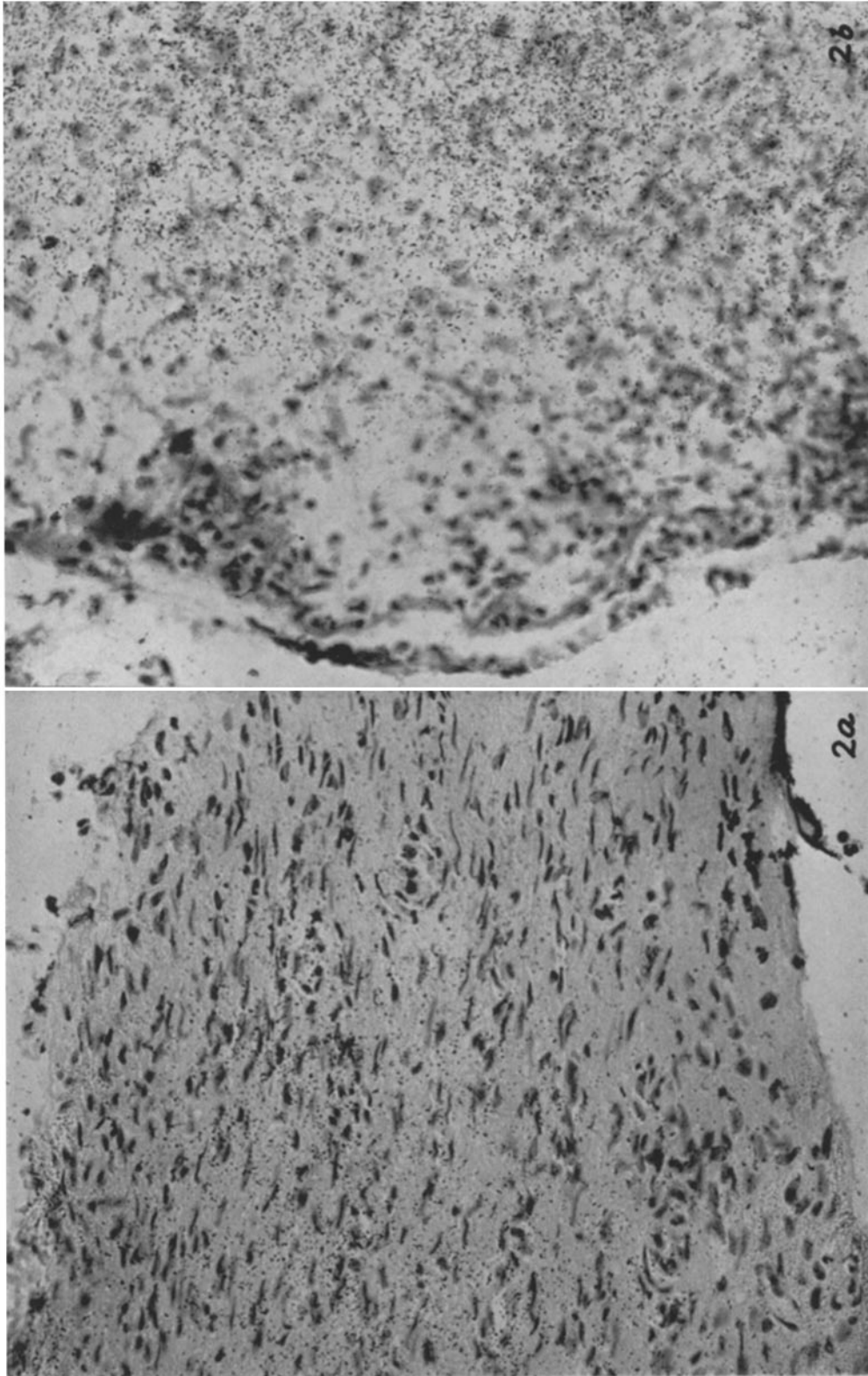
(Edwards and Udupa: Autoradiographic determination of S^{35})

FIG. 2. Floated autoradiograms of wounds showing the marginal loss of isotope in fluid-fixed tissues.

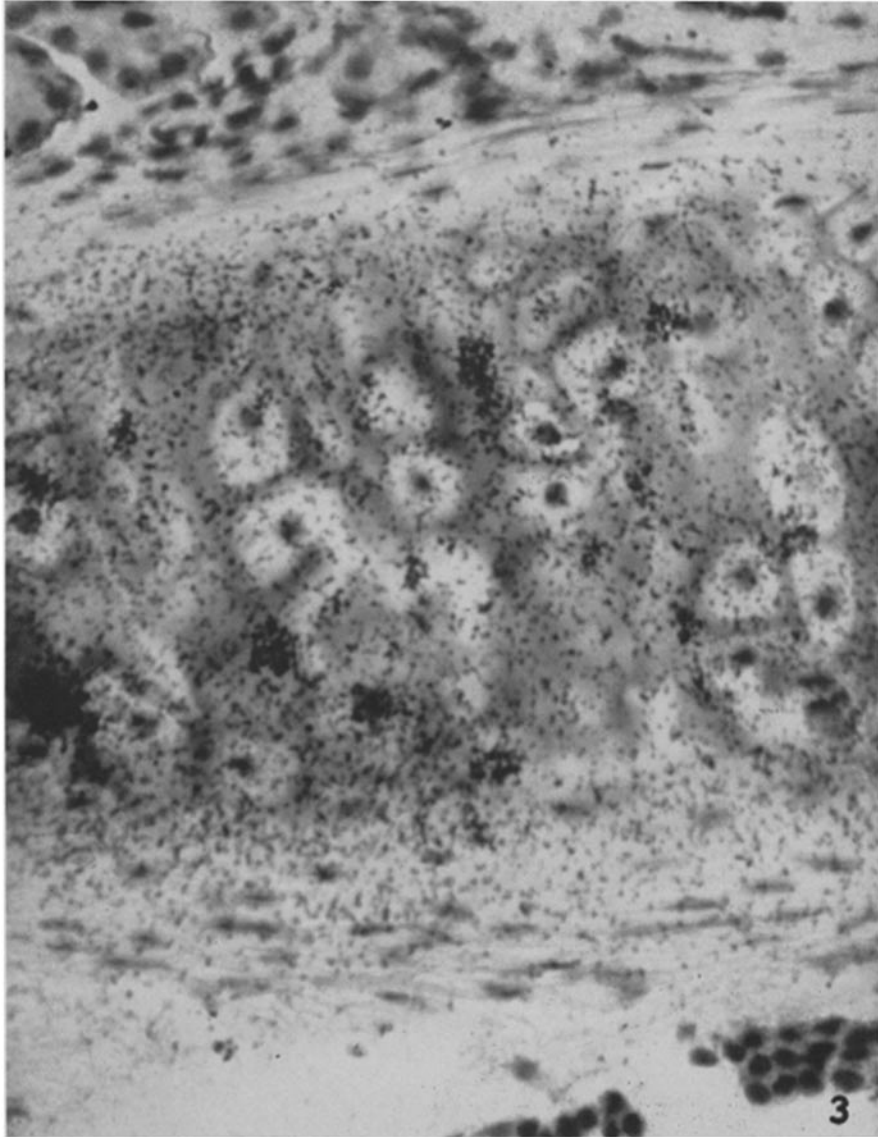FIG. 2 A. 6 micron section 100 hours after methionine S^{35}. Fixed in saturated aqueous lead subacetate.

FIG. 2 B. 6 micron section 12 hours after $Na_2S^{35}O_4$. Fixed in Carnoy's solution. Hematoxylin and dilute eosin. $\times$ 500.

(Edwards and Udupa: Autoradiographic determination of S^{35})

PLATE 245

FIG. 3. *Dry-mounted* autoradiogram of S^{35} (presumably chondroitin sulfate-S^{35}) in rat cartilage 100 hours after injection of 70 $\mu c./100$ gm. weight $Na_2S^{35}O_4$. NTB-3 emulsion (approximately 3 μ thickness); 16 weeks exposure. Hematoxylin and dilute eosin. $\times$ 1000.

(Edwards and Udupa: Autoradiographic determination of S^{35})