MOLECULAR PHARMACOLOGY, 10, 986–998

A Correlative Radioautographic, Fluorescent, and Histochemical Technique for Cytopharmacology

LLOYD J. ROTH, IHSAN M. DIAB, MITSUTOSHI WATANABE,[1] AND ROBERT J. DINERSTEIN

Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637

(Received June 24, 1974)

SUMMARY

ROTH, LLOYD J., DIAB, IHSAN M., WATANABE, MITSUTOSHI, AND DINERSTEIN, ROBERT J.: A correlative radioautographic, fluorescent, and histochemical technique for cytopharmacology. *Mol. Pharmacol.* **10**, 986–998 (1974).

Using mouse intestine, a model system has been developed in which two target cell loci, biogenic amine-containing enterochromaffin cells and regenerating crypt cells, are used for correlation of drug localization with fluorescence and microscopic structure. The combined procedure described for the cellular localization of drug and fluorophore is based on the use of frozen freeze-dried sections, dry-mounted on dried photographic emulsion, and has general applicability. Tissues need not be subjected to contact with any solvents until after radioautography, cellular fluorescence, and microspectrophotofluorometry have been completed. The use of freeze-dried frozen sections provides the possibility for localization by immunofluorescence labeling and thus offers the opportunity, by combining radioautographic and fluorescence labeling, to investigate competitive inhibition at the cellular level. Because this technique provides for reversible separation of the tissue from the emulsion for independent treatment, histochemical methods that are potentially destructive for the radioautogram can also be used. The correlative technique described here is rigorously controlled and systematic, and the results are unambiguous.

INTRODUCTION

The interaction of biologically active substances with specific cells and their constituents is a process which becomes pharmacologically significant when that interaction results in a response. The type of association which exists between drug and tissue depends on the chemical nature of the drug, the nature of the biological components, the type and stability of the association, and the chemical environment. It is manifest in the transient effects of many drugs that they exist only briefly at their site of action before they are removed, inactivated, or metabolized. The forces which bind the drug to specific sites are rarely irreversible. Rather, a combination of weaker binding forces (ionic, dipolar, hydrogen bonding, dispersion, hydrophobic, charge transfer) is generally assumed to be adequate to account for the drug-receptor complex. It cannot be overemphasized that the dissociation of drugs from binding sites is highly favored by the very nature of the associations which exist between the drug and the tissue (1–4).

This work was supported in part by Grants PHS 5-RO1 DA-00397, PHS 5-TO1 MH-07083, and PHS PO1 DA-00250 from the United States Public Health Service.

[1] Permanent address, Medical Research Laboratory, Central Institute for Experimental Animals, Kawasaki, Japan.

The purpose of this work has been to develop a general cytopharmacological method applicable to the localization of diffusible as well as covalently bound compounds. Such a goal imposes restraints on the use of any procedure likely to distort the localization of both endogenous and administered compounds. Treatment of the excised tissue is critical, and every effort must be made to guard against solution, translocation, and extraction. It is imperative, therefore, that all procedures potentially capable of displacing either endogenous or exogenous compounds be rigorously excluded from the methodology (5, 6).

Experimental procedures which employ polar or nonpolar solvents in the treatment of tissue clearly ignore the potential interaction between these solvents and weakly bound substances in the tissue, interactions which can result in translocation in and leaching from the specimen. Insolubility of the drug, when assumed adequate to protect against translocation of the substance during histological treatment, does not eliminate the possibility that the solvent may shift the drug within the specimen by suspension or desorption. Moreover, endogenous tissue components (e.g., biogenic amines) are likewise susceptible to the same solvent dislocating effects. In correlating drug localization with cellular concentrations of endogenous substances, the potential for erroneous cytochemical data is compounded when both drug and endogenous compound are diffusible.

A system based on the above criteria was developed previously in these laboratories for the radioautography of diffusible substances (7–13). Since all solvents were rigorously excluded in the radioautographic technique which we have described, that method could be used to verify the nuclear localization of [^{3}H]estradiol in cell fractionation studies. The radioautographic data confirmed the biochemical data, which were then used to formulate a theory for the interaction of hormones with receptor sites (14–16).

This report describes a model system, based on the use of frozen freeze-dried tissue sections, capable of general application for combined radioautography and fluorescence techniques. By reversibly separating the tissue sections from the emulsion, it is possible to perform radioautography, fluorescence microscopy, and histochemical staining independently or in sequence. The freeze-dried tissue can be protected from solvents, photographic solutions, viewing oil, and mounting media, while the photographic emulsion can be protected from histochemical stains which may alter or ablate the radioautogram. Treatment of the tissue with histochemical or immunological stains can be reserved for the penultimate step, before final staining.

METHODS

Tissue from mouse duodenal-jejunal area was selected for the model test system because two well-characterized target cell loci exist in the crypts, i.e., enterochromaffin cells containing biogenic amines, convertible by gaseous formaldehyde into fluorophore, and regenerating crypt cells whose nuclei can be readily labeled with [^{3}H]thymidine for radioautography.

Animals and Treatment

Four C576 male mice, weighing approximately 25 g, were fasted for 24 hr with water ad libitum. Tritiated thymidine (0.1 μg, 4 μCi/g of body weight) in 0.9% NaCl was injected intraperitoneally. After 1½ hr the animals received an intraperitoneal injection of 0.2 mg/g of dopa dissolved in 0.9% NaCl. One-half hour later the animals were killed by decapitation and the duodenal-jejunal tissue was rapidly excised for freezing at −210°.

Freezing and Sectioning of Tissue

Excised tissue (a block approximately 3 mm/side) is mounted on a brass tissue holder with tissue mince as an adhesive and quenched in an equilibrium mixture of solid-liquid nitrogen slush at −210°. The slush is prepared by evaporating liquid nitrogen from a Dewar flask in a desiccator under reduced pressure (11). Several round applicator sticks in the Dewar flask serve to prevent bumping. Continued evacuation, with a rotary vacuum pump, will convert all the

liquid to solid nitrogen. Superior conductivity with the slush is achieved because the tissue comes in contact with liquid nitrogen at the freezing point, where the heat of fusion rather than the latent heat of vaporization is utilized for freezing the tissue. Thus the formation of a gaseous insulator around the tissue is avoided. The frozen tissues mounted on the brass tissue holders may be stored in a liquid nitrogen refrigerator or placed in a cryostat for temperature equilibration in preparation for sectioning.

The cutting, in a cryostat, of frozen sections as thin as 0.5 μm and their subsequent freeze-drying has been described elsewhere (8, 9). The freeze-dried sections are transferred in an atmosphere of less than 15% relative humidity to a desiccator and stored under nitrogen.

Glass Assembly Procedure

Because treatment of tissue with water and organic solvents introduces the possibility for translocation or extraction of labeled compounds or biogenic amines in freeze-dried sections, a method for avoiding contact with solvents was required. A glass hinge system for accomplishing this objective is shown in Fig. 1. The two glass components of the assembly are similar to standard microscope slides and coverslips but differ significantly from the standard commercial products because they are made of the same composition optical glass having the same coefficient of expansion.

Combined radioautography and fluorescence microscopy is accomplished by dry-mounting freeze-dried sections on a plastic-coated glass slide and by bringing the sections into close contact with a glass coverslip coated with photographic emulsion. The tissue sections remain attached to the plastic-coated slide when the slide and coverslip, cemented together at one end, are parted. Care is taken to maintain the tissue at low humidity during development of the photographic emulsion by carrying out the process in a humidity-controlled darkroom. The developed emulsion on the spaced assembly is dried in a desiccator or air-dried in a low-humidity room for 1 hr, after which the tissue may be gassed with formaldehyde for development of the fluorophore. Reapposi-

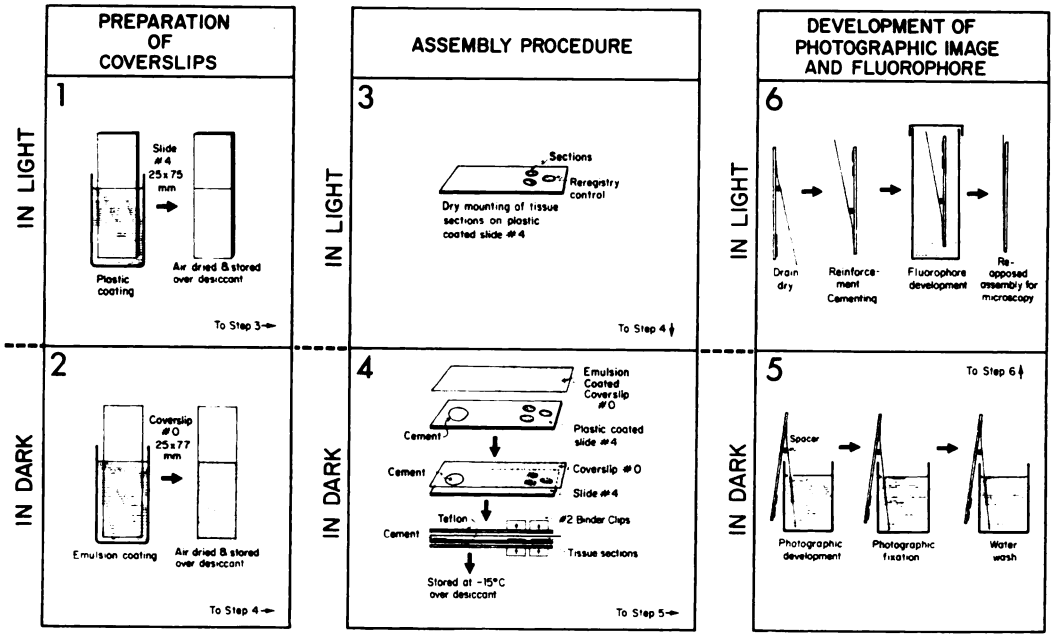


FIG. 1. *Glass assembly procedure*
See *Glass Assembly Procedure* under METHODS.

tion of tissue and radioautogram is accomplished by removing the spacer and closing the hinge with slight pressure. The radioautogram and the "fluorogram" are then viewed dry or under oil, which does not come in contact with the tissue.

Confirmation of reregistry is determined by microscopic examination of the reregistry control, described below, on the glass slide for realignment between the β-ray source and the radioautogram. The radioactive source may be either a plastic line or a tissue section labeled with covalently bound [^{3}H]thymidine.

Glass Assembly Procedure (Fig. 1)

Step 1. Clean No. 4 slides, 0.4 × 25 × 75 mm (Corning cover glass, No. 4 thickness), are dipped for two-thirds of their length into an Entellan[2] or Formvar[3] solution and then suspended for 3 hr in air to dry at room temperature. The terminal 2 mm of thickened, dried plastic is removed from both sides at the end of the No. 4 slide with a razor blade before tissue mounting.

Step 2. Photographic emulsion (Kodak NTB-3) is warmed to 40° in total darkness, poured into a 250-ml jar, and diluted 1:1 by the addition of deionized water at 40°. Clean No. 0 coverslips, 0.1 × 25 × 77 mm (Corning cover glass, No. 0 thickness), are dipped for two-thirds of their length into the emulsion for about 2 sec and suspended for drying overnight at a relative humidity of 60–80% in complete darkness. The dry emulsion-coated coverslips are then stored in sealed black slide boxes (Clay Adams) over Drierite for at least 24 hr at 4° before use. For background evaluation coverslips from each slide box are developed in D-19 developer (Kodak) for 4 min, washed, and fixed for 10 min at 15°. Developed emulsions which show a background less than 10 silver grains/1000 μm^2 are accepted for radioautography.

Step 3. In a temperature-controlled, low-humidity room (20°, 10–15% relative humidity) the freeze-dried sections, together with a reregistry control (discussed later), are transferred to a 2-cm^2 Teflon sheet. The sections are then positioned and smoothed out under the dissecting microscope. Using the Teflon sheet as a support, the sections are pressed against the dried, plastic-coated No. 4 slide. The sections adhere to the No. 4 slide, and the Teflon sheet falls off by simple inversion.

Step 4. In a low-humidity darkroom (10–15% relative humidity) a small drop of Eastman 910 adhesive (an alkyl-2-cyanoacrylate) is placed on the plastic-free glass end of the plastic-coated No. 4 slide. The emulsion-free end of the companion No. 0 coverslip is then apposed to the adhesive and both are aligned, with the emulsion-coated coverslip projecting 2 mm beyond the nonglued end of the assembly. After a few seconds the glued assembly is placed between two Teflon sheets (25 × 77 × 1 mm, Crane Packing Company, Morton Grove, Ill.) and held in place with four No. 2 binder clips (I.D.L. Manufacturing Company, Carlstadt, N. J.). The total assembly is then placed in light-tight boxes over Drierite for 2–6 hr before storage in a freezer at − 15° for radioautographic exposure.

Step 5. At the end of exposure time the sealed box, containing the complete assembly, is removed to a low-humidity darkroom for temperature equilibration: approximately 1 hr. The boxes are then opened in total darkness, the Teflon supports are removed, and a spacer is inserted between the hinged limbs of the assembly, with care taken not to touch the emulsion. The 2-mm protruding end of the emulsion-coated No. 0 coverslip serves as a finger grip to facilitate spacing the assembly. The emulsion is developed in the low-humidity room for 4 min in Kodak D-19 developer at 15°, fixed in Kodak acid fixer for 5 min at 15° and, washed in tap water for 10 min. Final drying is done in the low-humidity room for 1 hr.

Step 6. After drying, the hinged end of the glass assembly is dipped in Eastman 910 adhesive to reinforce the bond. When the adhesive is dry, the spaced assembly is transferred to a 100-ml Coplin jar containing 2 g of paraformaldehyde which has been

[2] Entellan solution is Entellan (E. Merck, Darmstadt) diluted 1:1 with xylene.

[3] Formvar solution is 2% Formvar (Monsanto) in ethylene dichloride.

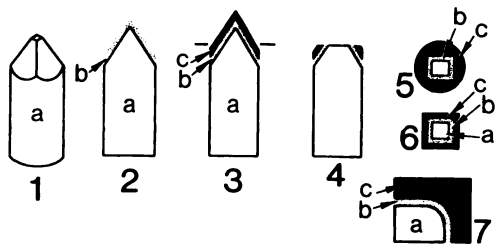


FIG. 2. *Plastic reregistry control*
See *Preparation of Plastic Reregistry Control* under METHODS. Order of assembly: *1*, epoxy plugs (*a*); *2*, radioactive film (*b*) over plug apex (*a*); *3*, epoxy cap (*c*) over radioactive film (*b*) on plug apex (*a*); *4*, truncated composite plug; *5*, vertical view of *4*; *6*, composite of plastic sections *a*, *b*, and *c*; *7*, quarter segment of *6*.

equilibrated under an ambient atmosphere of 50% relative humidity. The jar is then sealed and placed in an oven at 80° for 1 hr, after which it is cooled to room temperature. After heating at 80° in the presence of paraformaldehyde the radioautogram is not altered, as determined by pre- and post-microscopy. The glass assembly may be stored in the closed Coplin jar for later microscopic and fluorometric examination.

Preparation of Plastic Reregistry Control (Fig. 2)

A standard electron microscope BEEM (No. 00) capsule is filled with the following epoxy resin mixture: 7.0 parts Epon 812, 3.0 parts DDSA, 3.0 parts NMA, 0.2 parts DMP-30 Accelerator (Fisher Chemical Company), and 1.0 part Resoflex R296 (Cambridge Industries, Watertown, Mass.).

After curing at 60° in an oven for 24 hr the capsule is removed and cooled to room temperature. Then 3 mm of the butt end of the capsule are cut away, and the epoxy plug is removed. The plug is withdrawn from the capsule with tweezers, and the capsule is retained for a later procedure.

About 150 mg of polymerized [^{3}H]*n*-butyl methacrylate (Amersham/Searle; specific activity, 10 mCi/g) are dissolved in 10 ml of diethyl ether. Several drops of the ethereal solution are released onto a distilled water surface, which then spreads out as a thin film. The epoxy plug is lowered into the water beyond the periphery of the radioactive film. With the pointed end of the plug upward, the plug is raised under the film, as in stripping-film radioautography. The radioactive film is thus draped over the pointed end of the plug, while the excess is trimmed away from the plug with a razor blade. The radioactive film and plug are allowed to stand overnight.

The original BEEM capsule mold is punctured in the apex, and a small amount of commercial, room-temperature-setting epoxy (3-M Company) is placed in the bottom of the capsule. The plug, coated with radioactive methacrylate, is now reinserted into the capsule. Excess epoxy is extruded from the capsule via the hole in the tip, permitting a second layer of epoxy to form a cap over the radioactive methacrylate film.

After curing at room temperature for 24 hr the BEEM capsule is cut away from the composite epoxy plug. The composite plug is then mounted on a Sorvall MT-1 ultramicrotome, and the tip is cut off, forming a square. Sections of the composite are cut at room temperature at 0.75–1.0 μm with a glass knife. The plastic sections are transferred with tweezers to drops of water on No. 4 slides to be used in the correlative technique. Brief warming of the slide brings the plastic sections smoothly onto the surface of the No. 4 slide, where they serve as reregistry controls (Fig. 3).

Microscopy

Following development of the fluorophore in step 6 of Fig. 1 described above, the hinged glass assembly is reapposed without mounting media, and, with the thicker No. 4 glass slide on the bottom, is placed between the spring clamps and the base of the glass assembly holder (Fig. 4). A rubber "O" ring, ⅞ in. outer diameter, ¾ in. inner diameter, and 1/16 in. thick (Kirkhill Faucet, Kirkhill, Inc., Downey, Cal.), is cemented on the thin No. 0 coverslip, circumscribing the area to be examined. When cemented with Eastman 910 adhesive, the "O" ring serves to retain the immersion oil and prevent its seepage into the tissue.

Microscopic examination and photomicrography are performed with a Leitz Orthoplan microscope fitted with a 35-mm Leitz

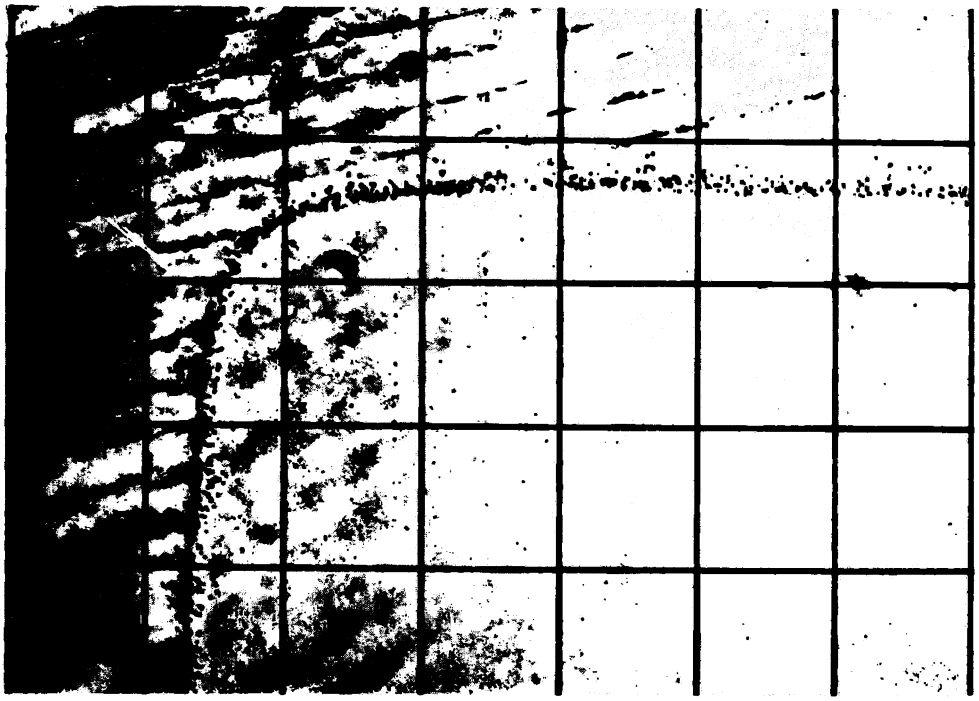


FIG. 3. *Radioautogram of reregistry control*
See *Reregistry Using Glass Hinge Assembly* under RESULTS.

camera body. In order to produce a grid pattern on the photographic image, a reticle (Leitz eyepiece net monochromator 10 × 10 mm, divided into 1.0-mm squares) is placed in an eyepiece in the vertical monocular photographic tube. The glass assembly is placed, without mounting media, between the spring clamps of the slide holder, which in turn is held in place by the microscope stage holder. The radioautogram is surveyed by focusing on the silver grains in the upper emulsion-coated coverslip, using tungsten light with a phase contrast condenser and a low-power dry objective. The tissue beneath may be seen by focusing the microscope at the level of the tissue on the slide. Repetition of this procedure over various portions of the slide provides a general impression of the radioautographic localization. Reregistry of the coverslip with the slide is also checked in this operation, together with a survey of tissue fluorescence under ultraviolet light. When the tissue areas to be photographed have been selected, the glass hinge assembly and its holder are locked in a fixed position by the microscope stage holder for tissue mapping, microspectrophotofluorometry, and photomicrography of tissue, fluorescence, and radioautography.

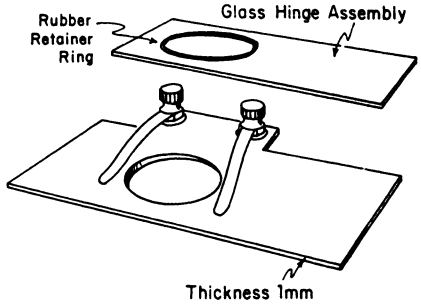


FIG. 4. *Glass hinge assembly holder*
See *Microscopy* under METHODS.

Tissue Map

A photographic grid map of the unstained tissue section is made at a series of magnifications, using dry objectives (4×, 25×, 40×) to assist in relocating specific areas under the microscope if the assembly should have to be removed from the stage, as, for

example, in final staining of the tissue. The grid is required for precise photographic superposition of tissue and fluorescence with the radioautogram (Fig. 7C).

Microspectrophotofluorometry

Fluorescence spectra of individual enterochromaffin cells are obtained using a microscope equipped with a Leitz Ploem vertical illuminator and with grating monochromators and optics (Schoeffel Instrument Corporation, Westwood, N. J.), assembled as follows for microspectrofluorometry. The light source for the microscope is a 300-W pressure xenon lamp (Illumination Industries PEK X-300 lamp) mounted in a Schoeffel LH 151 N lamp housing and powered by a Schoeffel LPS 255 lamp power supply. Light for fluorescence excitation is obtained by using the xenon lamp in combination with a grating monochromator (Schoeffel model GM 100; grating blazed for 240 nm; reciprocal linear dispersion, 8.5 nm/1.0 mm slit width) and then focusing the light for Ploem illumination. The Ploem illuminator is adjusted so as to use the TK 405 dichroic mirror, which provides excitation light in the 350–400 nm range and permits observation of emission fluorescence at wavelengths greater than 410 nm. The fluorescence from the specimen is analyzed by first isolating the light source in the microscopic field from the nonfluorescing background with a diaphragm system incorporated in a Schoeffel modified Leitz MPV microscope photometer attachment. The light then passes into a second, analyzing monochromator (Schoeffel model GM 100; grating blazed for 500 nm; reciprocal linear dispersion, 8.5 nm/1.0 mm slit width). The intensity of the light from the monochromator is measured with an EMI 9558B photomultiplier in a Schoeffel D-500 T housing equipped for both water and thermoelectric cooling (maximum cooling, −40°) to increase photomultiplier sensitivity. The picoammeter (Schoeffel model M 600) readings of the photomultiplier current are plotted vs. wavelength on a Houston HR89T *xy* recorder. Both the excitation and the emission monochromators are equipped with separate bidirectional motor drives (scanning the monochromators at a rate of 100 nm/min) coupled to potentiometers. As the wavelength setting of a monochromator is varied, the potentiometer varies the voltage supplied by a battery to change the x coordinate of the plotter. A switching system facilitates shifting between excitation and emission monochromators.

The tissue is surveyed for fluorescence using a 54× fluorite oil immersion objective. The cell to be scanned spectrophotometrically is centered in the microscopic field, and the slide is held in a fixed position for microspectrofluorometry and photomicrography. To obtain a spectrum of the fluorescing source in a tissue section, the area of interest in the center of the microscopic field (e.g., the cell) is first isolated from its surroundings with the MPV diaphragm system. Both the emission monochromator and the excitation

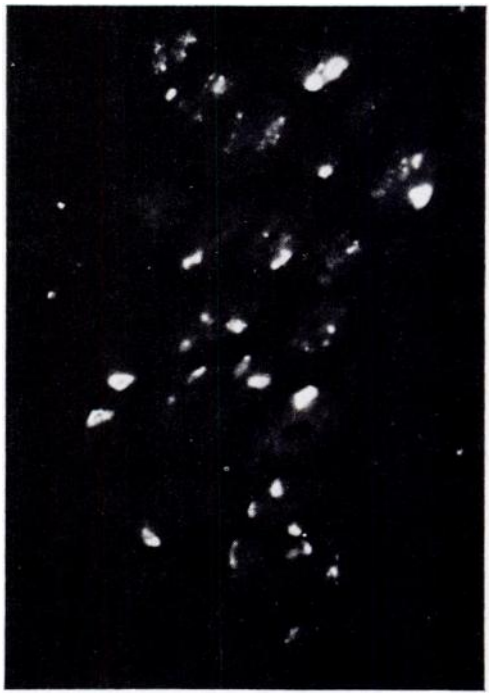


FIG. 5. *Fluorescent enterochromaffin cells in duodenal-jejunal tissue from mouse treated with 0.2 mg/g of dopa*

Frozen freeze-dried sections were dry-mounted on Entellan and gassed with formaldehyde, and the cellular fluorescence was photographed as described for microscopy procedures under METHODS. × 125.

monochromator are then adjusted manually until a maximum signal is registered on the picoammeter. With the emission monochromator setting fixed, an excitation spectrum is obtained by varying the excitation monochromator over a range from 340 to about 410 nm. The excitation monochromator is then set for the observed maximum in the excitation spectrum, and the emission monochromator is varied from about 420 nm until the emission peak is recorded. A complete uncorrected fluorescence spectrum (17, 18) with both excitation and emission peaks is thus obtained (Fig. 5).

Photomicrography of Tissue, Fluorescence, and Radioautography

To view tissue fluorescence a Ploem illuminator is used, because vertical illumination with ultraviolet light in conjunction with conventional tungsten substage illumination permits mixing of both. A drop of nonfluorescing immersion oil is placed within the retainer ring. Using an oil immersion objective (54×), the exact same area scanned for microspectrofluorescence, with the selected cell in the center of the field, is now photographed for silver grain distribution through the grid, using substage illumination with tungsten light and a phase contrast condenser. By switching to ultraviolet vertical illumination, with or without substage illumination, the tissue and its fluorescence may also be photographed. Thus a microspectrophotofluorescence scan is obtained together with photomicrographs of tissue, loci of fluorescence, and a radioautogram, each within a fixed grid. Data are obtained from the same area of tissue without moving the position of the slide assembly under the microscope or the condenser, with but a change of light source and focus.

Staining

The assembly is removed from the holder, and the glass hinge is separated with a spacer. The glass No. 4 slide carrying the tissue is dipped into a thin square Plexiglas container and stained with methyl green–pyronin for 5 min, after which it is washed twice in water, care taken to protect the developed emulsion from water and stain. Other histochemical stains may be used, because the tissue and the radioautogram are separated from each other. The spaced assembly is then air-dried for 1 hr. At this point a drop of mounting medium (Permount or oil) may be placed between the slide and the coverslip. The assembly is clamped in the slide holder, which is then replaced in the microscope stage slide holder. With the aid of the mapping photographs and vertical tube eyepiece grid, the same area of interest can be located and repositioned for simultaneous photography of the stained section and its radioautogram by focusing at a point between the two (Fig. 7D).

Printing

Precise realignment, by superposition of any combination of the above photographs, is achieved by realignment of the grid patterns using a Premier Multiprint easel (Photo Materials Company, Chicago).

RESULTS

Reregistry Using Glass Hinge Assembly

The 1.5-μm wide-line source of [^{3}H]*n*-butyl methacrylate within the composite plastic section (Fig. 2) was mounted on a No. 4 glass slide, which was processed for radioautography. Because the methacrylate and epoxy plastic have substantially different indices of refraction, the line source is readily distinguished by phase contrast microscopy. The bidirectional line of [^{3}H]*n*-butyl methacrylate serves as a control to demonstrate reregistry of the radioautogram on the thin No. 0 coverslip and the radioactive source on the No. 4 slide.

The accuracy of reregistry using the glass hinge procedure is shown in Fig. 3. By visual inspection the radioautogram overlies the bidirectional line source over its entire course. Using a standard scale (Zeiss micrometer with markings each 10 μm), the tritium-labeled line source was measured as 1.5 μm in width. By counting grains on each side of the line as a function of distance, a bell-shaped histogram was obtained, giving a "half-distance" as defined by Salpeter *et al.* (19, 20) of about 0.8 μm. Moreover, [^{3}H]thymidine radioautograms prepared by the glass

hinge technique exhibited similar distribution of silver grains, as seen with radioautograms prepared by the dipping technique.

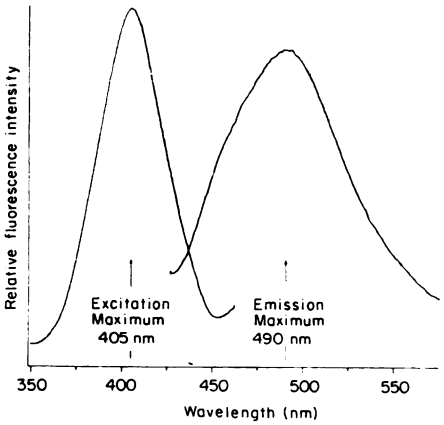
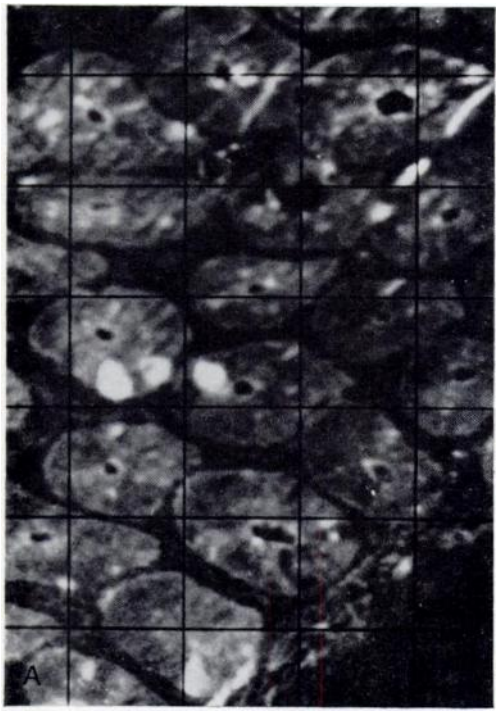


FIG. 6. *Microspectrofluorometric scan of an enterochromaffin cell*

See *Microspectrofluorometry* sections under METHODS and RESULTS.

It can be inferred that in the glass hinge technique the photographic emulsion is applied as closely to the radioactive source as it would be if the emulsion had been directly coated on the source itself.

To further demonstrate the accuracy of the glass hinge technique and to emphasize the importance of using the same composition of glass for coverslip and slide, an experiment was performed using two different types of glass in the slide and coverslip. A commercial glass slide (coefficient of expansion, 91 $\times$ 10^{-7}/°C) and a glass coverslip (coefficient of expansion, 72 $\times$ 10^{-7}/°C) were assembled into a hinge with the plastic radioactive line source. The assembly was exposed at 0°, and the emulsion was developed. When viewed at 25°, the radioactive line had shifted away from the plastic line in the direction toward the cemented end. The line source was offset from the mean position of the silver grains by some



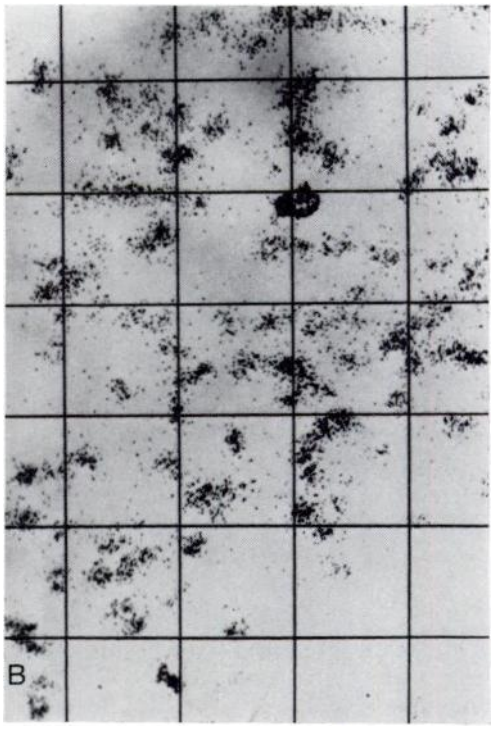


FIG. 7A. Photomicrograph of intestinal crypts and fluorescing enterochromaffin cells. See *Superposition of Radioautogram, Tissue, and Cellular Fluorescence* under RESULTS. $\times$ 275.

FIG. 7B. Radioautographic image of [^{3}H]thymidine from intestinal crypts. See *Superposition of Radioautogram, Tissue, and Cellular Fluorescence* under RESULTS. $\times$ 275.

2.5 μm. This difference is about the expected value of 3 μm calculated from the difference in expansion for the commercial 75-mm coverslip and 75-mm glass slide when warmed from 0° to 25°. The glass hinge technique is thus sensitive enough to detect the difference in the coefficients of expansion for these two types of glass.

Microspectrophotofluorometry

Enterochromaffin cells were observed in the intestines of mice given L-dopa (2 mg/g) 1½ hr before death (Fig. 5). Fluorescent spectra were obtained of enterochromaffin cells, using the microspectrophotofluorometer equipment described above. A typical spectrum of one such cell is shown in Fig. 6. Comparison of this uncorrected spectrum with standard spectra of norepinephrine and serotonin in bovine albumin droplets (17) suggests that the observed spectrum is the sum of both incorporated catecholamine derived from L-dopa and endogenous 5-hydroxytryptamine in the enterochromaffin cell (21).

Superposition of Radioautogram, Tissue, and Cellular Fluorescence

The photomicrograph of the tissue and fluorescing enterochromaffin cells (Fig. 7A) was combined with the radioautographic image (Fig. 7B) by superimposing the grid pattern of each on a blank control grid mounted on the Multiprint easel. The superposition print of tissue fluorescence and radioautogram is shown in Fig. 7C. Reregistry was demonstrated as described for Fig. 3 and by the coincidence of [^{3}H]thymidine labeling with nuclei of the intestinal crypt cells. Finally, the section on the glass assembly was stained with an aqueous solution of methyl green–pyronin, washed, air-dried, and permanently mounted with a drop of Permount between the No. 0 coverslip and the

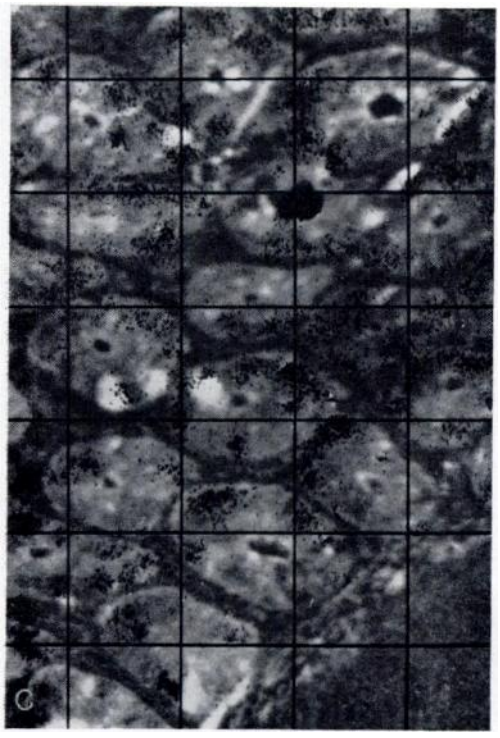


FIG. 7C. Combined radioautogram and fluorogram. See *Superposition of Radioautogram, Tissue, and Cellular Fluorescence* under RESULTS. × 275.

FIG. 7D. Radioautogram of stained intestinal crypts. See *Superposition of Radioautogram, Tissue, and Cellular Fluorescence* under RESULTS. × 275.

No. 4 slide (Fig. 7D). With Formvar-coated slides it was possible to treat mouse intestinal tissue with a periodic acid–Schiff stain, because the tissue is separated from the emulsion without shifting or loosening of the Formvar plastic base on the slide; the base is unstable when this stain is applied to tissue mounted on Entellan-coated slides.

Diffusion and Stability of Fluorophore

Frozen freeze-dried sections of mouse intestine were dry-mounted on Entellan-coated slides to test the effect of humidity on the location of biogenic amines in intestinal crypt cells. Freeze-dried tissues maintained in an atmosphere of low relative humidity (less than 15%) before treatment with formaldehyde gas showed no apparent diffusion. Similar sections exposed to more than 50% humidity for 15 min and dried over Drierite, followed by gassing, showed diffusion throughout the entire crypt. Furthermore, exposure to such humidity resulted in disappearance of fluorescence, presumably because of oxidation. This loss, but not diffusion, was prevented when a dilute aqueous solution of ascorbic acid was applied to the tissue before drying. When frozen freeze-dried sections of mouse intestines were dry-mounted on plastic-coated slides and gassed with formaldehyde the majority of the fluorescent cells showed a substantial loss in intensity following passage through photographic solutions, while some disappeared entirely.

DISCUSSION

The localization of drugs in tissue at the cellular level represents an important procedure for studying the mechanisms by which drugs act (14–16, 22–24). Radioautography can be a significant tool in such determinations, provided that the methodology is systematically controlled and capable of reliably establishing localization.

The use of frozen freeze-dried, dry-mounted sections on dried photographic emulsion seems to be the only method for avoiding the problem of extraction and translocation inherent in the use of conventional histological techniques (5). The fact that aqueous processing of tissue for radioautography carries a serious risk of dislocating water-soluble substances during histological processing has been appreciated for some time (25–30). Solvent effects of nonaqueous fluids, such as hot paraffin, unpolymerized plastic, and nonpolar organic solvents, have also been pointed out (31–34).

Fluorescence microscopy has been widely used for studying the localization of biogenic amines after it was found that they were converted by formaldehyde gas to the corresponding fluorophore in the presence of protein and in freeze-dried tissue (35). The use of frozen, freeze-dried, dry-mounted tissue sections for biogenic amine fluorescence microscopy has been successfully applied here without resorting to liquid fixation of tissue, embedding, de-embedding, or other solvent application. The combined techniques of fluorescence microscopy and radioautography are based on a series of sequential steps, from which it follows that any single link, improperly designed or executed, may lead to errors sufficient to invalidate all the data so derived.

Tissue fixation has sometimes been used to prevent translocation and/or extraction, usually on the grounds that an insoluble fluorophore *in situ* can be induced with tissue components. A number of investigators have studied the possibility of combining radioautography with fluorescence microscopy (36–44). In each case, however, methods were used which allowed histological agents or photographic developing solutions to come in contact with the tissue specimen. The assumption that catecholamine-induced fluorophores are immobilized in tissue by treatment with formaldehyde gas has been found to be generally inapplicable. Consequently Bjorklund and Falck (45) have recommended, when exploring new tissue sites for monoamines, that the exposure of tissue to hot paraffin or organic solvents be avoided. Thus dependence on such immobilization must be restricted to rigorously proven cases. Gaseous treatment with formaldehyde does not necessarily prevent loss of fluorophore when the tissue is exposed to wet photographic emulsion. In our experiments with duodenal–jejunal crypt tissue gaseous formaldehyde treatment of frozen freeze-dried tissue sections provided inadequate protection against loss of fluoro-

phore by passage through water or photographic solutions. The use of wet liquid emulsion and wet stripping film is likewise contraindicated.

The solvent-free system which we have described can meet the criteria of high solubility and little or no binding because the methodology avoids all agents and procedures likely to result in translocation or extraction. Cellular correlation studies can be carried out concomitantly or by superposition photography. We also point out that the option for localization of drugs by immunofluorescence is retained in the procedure because the tissue, prior to final histochemical staining, is free from contact with solvents. Assuming that the antigen in question has not been denatured by exposure to formaldehyde gas, the freeze-dried specimen should be suitable for immunofluorescence studies in combination with radioautography and induced biogenic amine fluorescence. In the event that formaldehyde is found to be contraindicated for immunofluorescence, the induction of endogenous fluorophores by formaldehyde gas may be omitted, leaving the ability to compare radioautographic and immunofluorescent localization without reference to cells containing endogenous biogenic amines.

The methodology discussed is combined or segmental, in that it provides for (a) examination by phase contrast microscopy of frozen freeze-dried sections untreated by media or solvents, (b) radioautography of diffusible and nondiffusible substances, (c) fluorescence microscopy and microspectrophotofluorometry, (d) immunofluorescence, and (e) final histochemical staining of the tissue isolated from the photographic emulsion. This last procedure makes possible, for the first time, the use of such tissue stains as periodic acid–Schiff, which ablate the silver grains (34, 39, 46) when it comes into contact with the developed emulsion.

Relative binding and the identification of drugs and biogenic amines may be ascertained when the results are compared with various histological procedures utilizing solvents which wash out unbound constituents (10, 47). The effect of releasing agents or biogenic amines may be examined in a similar manner. Competitive antagonism is now open to investigation, in which one component can be radioactively labeled while the other either fluoresces or is stained immunologically. The latter is made feasible because the section will have remained untreated to this point. Correlations among fluorescence, radioautography, and histochemical staining may also be assessed.

CONCLUSIONS

High-resolution radioautography is the only research technique currently available for the direct examination of labeled cells in a heterogeneous population. As such it has become a valuable tool for studying the localization of drugs without disrupting the structural integrity of the system under investigation. Selective histochemical staining without alteration of the radioautogram provides an added dimension for cell identification. Correlations existing between labeled drug and biogenic amine-containing cells can now be carried out: for example, correlative studies on the localization of [^{3}H]-lysergic acid diethylamide and serotonin-containing neurons. Studies of the cytological relationships between drugs detected by radioautography and immunofluorescence labeling, for example, ^{3}H-labeled narcotic agonist and fluorescent antibody-labeled antagonist, seem a distinct possibility.

The use of dry-mounted, frozen freeze-dried tissue sections, reversibly separable from the photographic emulsion, permits isolated development of the radioautogram, induction of fluorescence, microspectrofluorometry of single cells, and final histochemical staining of the same tissue section. Simplicity consistent with accuracy was the guideline controlling the development of the method, whereby the final result obtained depends on the rigor applied to each step in the procedure. The technique is systematic, each step is independently justifiable, and the results are unambiguous.

REFERENCES

1. Goldstein, A., Aronow, L. & Kalman, S. M. (1974) in *Principles of Drug Action: The Basis of Pharmacology*, Ed. 2, pp. 2–22, Wiley, New York.

2. Paton, W. D. M. (1970) *Ciba Found. Symp. Molecular Properties of Drug Receptors*, (Porter, R. & O'Conner, M., eds.), 3-32.
3. Burgen, A. S. V. (1970) *Ciba Found. Symp. Molecular Properties of Drug Receptors*, (Porter R. & O'Connor, M., eds.), 163-164, 263-269.
4. Watson, J. D. (1970) in *Molecular Biology of the Gene*, Ed. 2, pp. 102-140, Benjamin, New York.
5. Roth, L. J. (1971) in *Handbook of Experimental Pharmacology, New Series* (Brodie, B. B. & Gillette, J., eds.), Vol. 28, pp. 286-316, Springer, Berlin.
6. Roth, L. J., Diab, I. M., Asghar, K. & Dinerstein, R. J. (1972) *Fed. Proc.*, **31**, 579.
7. Roth, L. J. & Stumpf, W. E. (1969) *Autoradiography of Diffusible Substances*, Academic Press, New York.
8. Stumpf, W. E. & Roth, L. J. (1966) *J. Histochem. Cytochem.*, **14**, 274-287.
9. Stumpf, W. E. & Roth, L. J. (1967) *J. Histochem. Cytochem.*, 15- 243-251.
10. Diab, I. M. & Roth, L. J. (1970) *Stain Technol.*, **45**, 285-291.
11. Diab, I. M. & Roth, L. J. (1972) *J. Microsc. (Oxf.)*, **96**, 155-164.
12. Diab, I. M. & Roth, L. J. (1972) *J. Reticuloendothel. Soc.*, **12**, 503-513.
13. Brown, D. A., Stumpf, W. E. & Roth, L. J. (1969) *J. Cell Sci.* **4**, 265-288.
14. Jensen, E. V., DeSombre, E. R., Jungblut, P. W., Stumpf, W. E. & Roth, L. J. (1971) in *Autoradiography of Diffusible Substances* (Roth, L. J. & Stumpf, W. E., eds.), pp. 81-97, Academic Press, New York.
15. O'Malley, B. W. & Means, A. R. (1974) *Science*, **183**, 610-620.
16. Jensen, E. V. & DeSombre, E. R. (1973) *Science*, **182**, 126-134.
17. Van Orden, L. S., III (1970) *Biochem. Pharmacol.*, **19**, 1105-1117.
18. Ritzen, M. (1967) M.D. thesis, Karolinska Institutet, Stockholm.
19. Salpeter, M. M. (1969) in *Autoradiography of Diffusible Substances* (Roth, L. J. & Stumpf, W. E., eds.), pp. 335-348, Academic Press, New York.
20. Salpeter, M. M., Bachmann, L. & Salpeter, E. E. (1969) *J. Cell Biol.*, **41**, 1-20.
21. Hakanson, R., Owman, C., Sjoberg, N. O. & Sporrong, B. (1970) *Histochemie*, **21**, 189-220.
22. Waser, P. G. (1967) *Ann. N. Y. Acad. Sci.*, **144**, 737-755.
23. Rogers, A. W., Darzynkiewicz, Z., Salpeter, M. M., Ostrowski, K. & Barnard, E. A. (1969) *J. Cell Biol.*, **41**, 665-685.
24. Salpeter, M. M. (1969) *J. Cell Biol.* **42**, 122-134.
25. Ullberg, S. (1958) *Proc. 2nd U. N. Int. Conf. Peaceful Uses of Atomic Energy*, Vol. 24, *Isotopes in Biochemistry and Physiology*, Pt I., pp. 248-254, United Nations, Geneva.
26. Fitzgerald, P. J. (1961) *Lab. Invest.*, **10**, 846-856.
27. Hammarstrom, L., Appelgren, L. E. & Ullberg, S. (1965) *Exp. Cell Res.*, **37**, 608-613.
28. Appelton, T. C. (1966) *J. Histochem. Cytochem.*, **14**, 414-420.
29. Christensen, A. K. (1971) *J. Cell Biol.*, **51**, 772-804.
30. Appelton, T. C. (1974) *J. Microsc. (Oxf.)*, **100**, 49-74.
31. Hutchinson, T. E., Bacaner, M., Broadhurst, J. & Lilley, J. (1974) *Rev. Sci. Instrum.*, **45**, 252-255.
32. Richter, C. P. & Warner, C. L. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 598-601.
33. Pease, D. C. (1964) *Histological Techniques for Electron Microscopy*, Ed. 2, p. 84, Academic Press, New York.
34. Rogers, A. W. (1969) *Techniques of Autoradiography*, p. 198, Elsevier, Amsterdam.
35. Bjorklund, A., Falck, B., Lindvall, O. & Svensson, L.-Å. (1973) *J. Histochem. Cytochem.*, **21**, 17-25.
36. Ritzen, M., Hammarstrom, L. & Ullberg, S. (1965) *Biochem. Pharmacol.*, **14**, 313-321.
37. Falck, B. & Owman, C. (1965) *Acta. Univ. Lund., Sec. II*, No. 7, 1-23.
38. Hammarstrom, L., Ritzen, M. & Ullberg, S. (1966) *Experientia*, **22**, 213-215.
39. Tjalve, H. (1971) *Acta. Physiol. Scand., Suppl.* **360**, 1-122.
40. Winckler, J. & Hempel, K. (1971) *Histochemie*, **25**, 130-142.
41. Hokfelt, T. & Ljungdahl, A. (1971) *Prog. Brain Res.*, **34**, 88-102.
42. Masuoka, D. T. & Placidi, G. F. (1970) *J. Histochem. Cytochem.*, **18**, 660-666.
43. Masuoka, D. T., Placidi, G. F. & Gosling, J. A. (1971) *Prog. Brain Res.*, **34**, 77-86.
44. Grant, L. D. & Stumpf, W. E. (1973) *J. Histochem. Cytochem.*, **23**, 404.
45. Bjorklund, A. & Falck, B. (1968) *J. Histochem. Cytochem.*, **16**, 717-720.
46. Sar, M. & Stumpf, W. E. (1973) *Science*, **179**, 389-391.
47. Diab, I. M., Freedman, D. X. & Roth, L. J. (1971) *Science*, **173**, 1022-1024.