The filtrates from the more concentrated infusions in basal medium are excellent growth mediums for the tubercle bacillus (Table I). If more dilute infusions (*e.g.*, 1 part or less of yolk in 100 parts of basal medium) are made, very little of the growth stimulating material goes into solution and the filtrates do not support growth well.

These filtrates, while free from gross particles, are opalescent emulsions. An effort was made to secure a yolk filtrate that was water clear and would yet support vigorous growth of small inocula. This was done by extracting egg yolk repeatedly with acetone at room temperature; drying and powdering the residue; then making an infusion of the powder (0.66 g in 25 ml of basal medium) and filtering through a Coors filter. The filtrate was water clear. With an inoculum of 10^{-4} mg, smears were positive in 5 days and heavily positive in 8 days. With an inoculum of 10^{-8} mg smears were positive in 18 days and growth was heavy in 25 days. When the acetone-extracted yolk was further extracted with hot alcohol (which removes the phospholipids), the aqueous extract of the residue did not support the growth of tubercle bacilli. The fraction removed by the hot alcohol, when added to basal medium and autoclaved, did support growth. That the phospholipid fraction of egg yolk is largely responsible for the extraordinary capacity of the yolk to stimulate the growth of tubercle bacilli is clear from the reported observations of Boissevain and Schultz(8), Dubos(9), Finlayson(10), and others. Sphingomyelin, which constitutes about 0.3% of the weight of the yolk, is known to enhance the growth of the tubercle bacillus(11). Experiments still in progress indicate that other constituents besides the phospholipids may be equally important.

Summary. 1. Egg yolk can be used as the basis for several easily prepared liquid and semi-liquid mediums.

2. On such mediums, growth of the tubercle bacillus is rapid and heavy, even from small inocula.

3. By first removing the acetone soluble fats, water clear extracts of egg yolk can be prepared; these will support growth from small inocula.

8. Boissevain, C. H., and Schultz, H. W., *Am. Rev. Tuberc.*, 1938, v38, 624.

9. Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 361.

10. Finlayson, M. K., *J. Path. Bact.*, 1946, v58, 88.

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A Radioautographic Method for Detailed Localization of Radioactive Isotopes in Tissues without Isotope Loss. (17739)

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Animal tissues are prepared for radioautographs by freezing in isopentane at –170°C followed by vacuum dehydration, infiltration with paraffin under vacuum, cutting with the technic described previously(1), and direct mounting of the section on a microscope slide or a thin cover glass. The tissue holder (slide or cover glass) is then placed in a vertical position in an oven at approximately 45°C; the paraffin liquefies and all exceess paraffin is drained from the slide and tissue section. An alternative method is to remove the paraffin more completely by means of a paraffin solvent such as xylol, warm dioxane, or warm absolute alcohol. The tissue is returned to the oven for a suitable length of time to ensure complete evaporation of the

1. Holt, M. W., Cowing, R. F., and Warren, S., *Science*, 1949, v110, 328.

paraffin solvent (if one is used) before the section is attached to the photographic plate. The results obtained to date have indicated that use of the above-mentioned paraffin solvents does not produce appreciable variations in radio-autographs obtained from frozen-dehydrated tissues with I^{131}. This is being investigated further. As was reported previously(1) for frozen-dehydrated tissues with P^{32}, placing the tissue containing I^{131} in water does result in loss of a major portion of the activity. These results have been obtained for varying time intervals (such as 2, 4, 24, and 48 hours) from injection of the isotope to killing the animal.

The photographic exposure is made by clamping the tissue slide or cover glass face to face with the emulsion side of a photographic plate. In order to prevent direct contact of the tissue with the emulsion surface a thin plastic "spacer" is used. This is a ring of polystyrene 1 mil in thickness which encircles the tissue section; the diameter of the opening is approximately 1 cm. The rings are cut from sheet polystyrene by means of a special punch devised for this purpose. Since the tissue is ordinarily cut at a thickness of 8 μ, the use of the plastic spacer provides an air gap of approximately 18 μ between the tissue surface and the emulsion. The spacer is considered to be desirable because it eliminates the possibility of direct chemical action of the tissue on the emulsion, and it permits easy separation of the two slides at the end of the exposure. The tissue slide or cover glass and photographic plate are clamped together (using small binder clips or other suitable clamps) and stored for exposure. As will be pointed out later, mounting the tissue sections on a cover glass is preferable if localizations are to be studied at high magnifications. After a suitable exposure time, the two slides are separated; the photographic plate is developed and the tissue slide is stained. The tissue slide and radioautograph are both examined under the microscope and photomicrographs are made of the corresponding images in areas of interest. The eyepiece of the microscope is fitted with a ruled micrometer disc which is adjusted in position to give a sharp focus at the photographic plate.

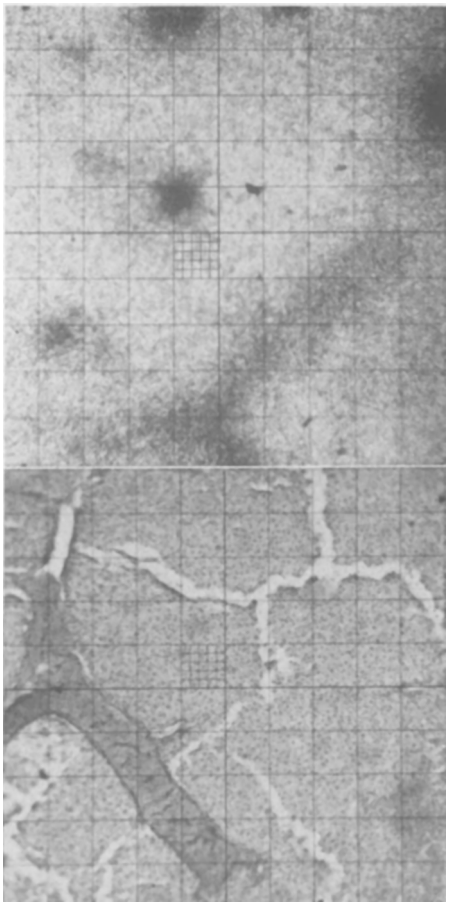

FIG. 1.

a (bottom), Hematoxylin stained 8 μ section of frozen dehydrated liver from a 135 g rat injected with 48 μc carrier free I^{131} 3 hours before sacrifice; *b* (top), corresponding radioautograph on Eastman Kodak NTB-3 50 μ plate, 17-day exposure started 12 days after the injection. Magnification 50×.

The photographs illustrate the method used in locating regions of the tissue that correspond to numerous as yet unexplained "hot spots" observed in the liver of this animal.

The coordinate ruling, which photographs with the tissue image, is very useful in matching corresponding regions of the two resulting photographs (Fig. 1). In locating corresponding positions on tissue and radioautograph slides, it is necessary to have a coincident reference mark on the two slides which can be either within the field to be photographed or outside. The use of a reference mark within the field is illustrated by the large blood vessel shown in Fig. 1. The procedure used was

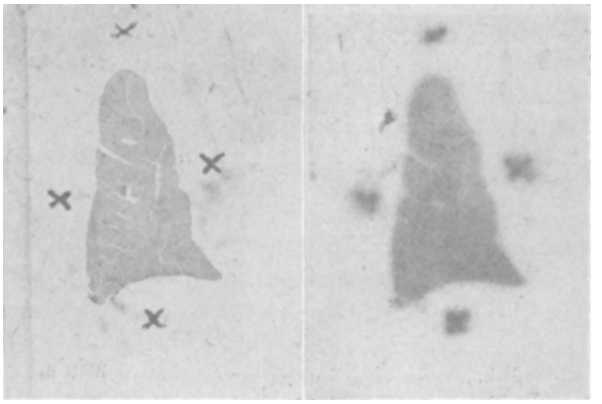

FIG. 2.

a (left), Hematoxylin stained section (8 μ) of frozen dehydrated liver from a 122 g rat injected with 500 μc carrier P^{32} two hours before sacrifice. *b* (right), Corresponding radioautograph on Eastman Kodak NTB-3 50 μ plate, 21-hour exposure started 8 days after the injection. X marks made with ink containing 4.4 μc P^{32}/cc. Magnification 3×.

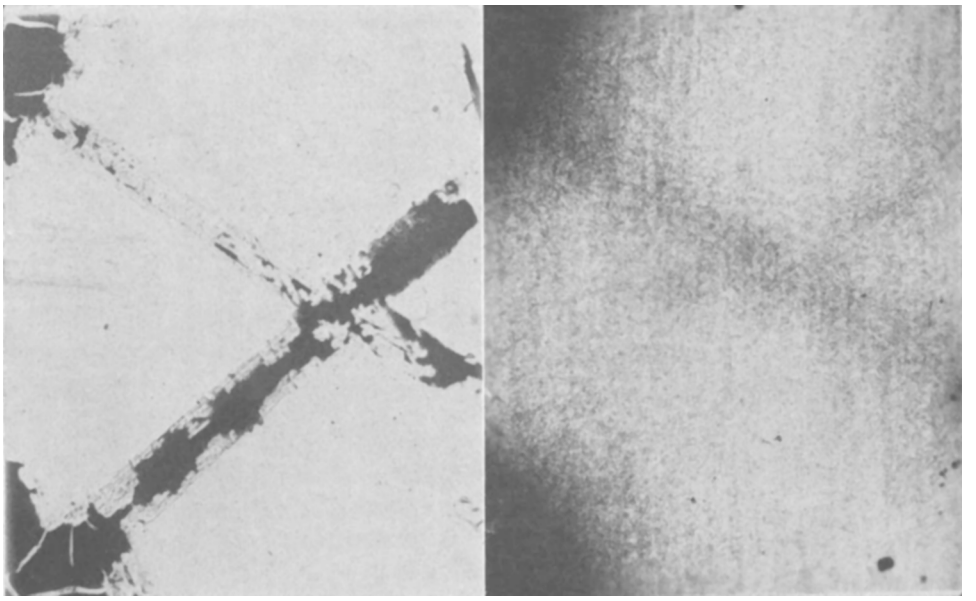

FIG. 3.

a (left), A very small ink X mark which was made by placing two dots of ink on the slide and pulling an extremely fine glass capillary through the ink dot and along the slide before the ink in the dot had been allowed to dry. With this method, lines approximately 50 μ wide can be made. *b* (right), Radioautograph obtained from the ink mark illustrated in *a* shown as a mirror image. The ink used contained approximately 200 μc P^{32}/cc and the exposure time with NTB-3 plates was 3 hours. Magnification 50×.

to adjust the position of the reference mark on each slide to coincide with a definite position on the eyepiece ruling before photographing.

With high magnifications, it is necessary to use reference marks located outside the microscope field. This can be accomplished by employing a calibrated mechanical stage on the microscope in combination with either the ocular micrometer ruling or a cross-hair in the

observation eyepiece of the photomicrographic camera. The reference mark is centered with the cross-hair or micrometer ruling and the position of the slide noted; the slide is then moved to the object to be photographed and the procedure repeated. The reference mark on the second slide is then located, the slide is moved to the position corresponding to that of the object photographed on the first slide (as determined by the calibrated stage) and this region is photographed.

In order to mark slides, a radioactive ink has been developed. The ink is prepared by adding an appropriate amount of carrier free P^{32} to glass marking ink (Clay-Adams Gold Seal black ink was used). Photographs showing the type of marks used and the corresponding radio-autographs are shown in Fig. 2 and 3; a description of the method used in preparing the marks is included under the figure caption. The precision of this method has been tested by using one of the ink marks as a reference mark and locating the position of other marks on the slide and radioautograph. It was found that the accuracy in determining the position of the marks on the radioautographs with respect to one another and to the tissue slide marks was as good as the accuracy of the mechanical stages tested (to within 100 μ with an ordinary mechanical stage and to within 25 μ using a modified mechanical stage with this degree of precision).

Since the radioautograph obtained with this technic is a mirror image of the tissue section, it is usually desirable to photograph the tissue section upside down(2). This makes the images correspond and simplifies comparisons of the photomicrographs. In order to focus on the inverted tissue section at high magnifications, it is necessary for the section to be mounted on a thin cover glass. After the photographic exposure has been made and the sections have been stained, the cover glass with attached sections is mounted with balsam on an ordinary slide. In this way, mirror images are avoided, and when the slides are observed in the usual manner the orientation of the tissue sections corresponds directly to the radioautographs.

2. Boyd, G. A., MDDC, 1226.

In making further refinements of the method, the following factors will determine the accuracy to be attained: (1) limitations due to the size of the reference marks used; (2) the accuracy of the mechanical stage (further refinement can readily increase this precision to ± 2 μ); (3) the distance separating the tissue and the emulsion surface; and (4) the resolution of the emulsion (an emulsion thickness of around 10 μ or less should improve this factor). It would also be desirable to decrease the thickness of the tissue section and to minimize back-scattering from the slide surfaces.

The basic principle of the method of making radioautographic exposures described in this paper (*i.e.* facing the specimen to the photographic plate followed by separation of the specimen and the plate at the end of the exposure) was originated by the work of London(3) and of Lacassagne and Lattes(4) and has been used in many studies. The primary objections to the method have been due to lack of sharpness of the radioautographs and to difficulties in matching the radioautograph with histological details(5). A method of matching the tissue slide and radioautograph which is somewhat similar to the one described here has been reported previously by Boyd (2). This technic employs a double microscope comparator with a mechanical stage common to two microscopes. A reference mark is prepared by taking a shadowgraph of an 8-10 μ piece of gold leaf which is placed near the tissue section.

The procedure of preparing radioautographs by painting the tissue section with liquid photographic emulsion, as described by Belanger and Leblond(6) was tried in this laboratory. Some difficulties were experienced with this method (such as those mentioned by Evans(5)); also, in working with frozen-dehydrated tissues with isotope concentrations

3. London, E. S., *Arch. d'electric. med.*, 1904, v12, 363.

4. Lacassagne, M. A., and Lattes, J. S., *C. R. Acad. d. sc.*, 1924, v178, 488.

5. Evans, T. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 313.

6. Belanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, v39, 8.

comparable to those reported previously(1), a blurred radioautograph resulted. This was due to loss of the isotope into the liquid emulsion. The method described by Endicott(7) and Evans(5) of permanently mounting the tissue section directly on the photographic emulsion offers the advantage of intimate contact of the tissue section and the emulsion with corresponding gain in resolution. However, the question of possible chemical fogging of the emulsion by the tissue does arise. This method is also subject to staining difficulties. Conventional mounting technics are employed (floating the tissue on water) and it would not be easily adaptable to the cutting and mounting technic developed in this laboratory for avoiding isotope loss because of the problem of removing the paraffin.

The use of stripping film(8,9) for preparing radioautographs avoids staining difficulties when the tissue is mounted on the photographically insensitive side. However, it is more complicated to handle than an ordinary slide; the tissue must be mounted in the darkroom; and the tissue is separated from the emulsion by a 10 μ cellulose ester (which probably results in more absorption and scattering of radiation than would be produced by 18 μ of air). It does have the advantage of minimizing back-scattering.

Although there is partial separation of the tissue image and the radioautograph because of difference in the focal plane in the stripping film method, these latter 3 methods all have the disadvantage of super-position of images. This makes it difficult to see details of either one individually, especially where the tissue section is stained.

Summary. The method described in this paper incorporates the following desirable features:

1. It is possible to prepare radioautographs from tissue that has been frozen and dehydrated in vacuum and mounted by procedures that avoid contact of the tissue with reagents that would extract the radioactive material.

2. Sharp images are obtained; yet the method avoids possible difficulties due to direct chemical contact of the tissue with the photographic emulsion.

3. There are no difficulties in staining and developing, since the two slides are handled separately.

4. Due to the fact that two images are on separate slides, the photographic image formed does not obscure structural details of the tissue section or vice versa; comparisons are always made on the same tissue section that was used in making the photographic exposure.

5. It is possible to match localizations accurately on the two slides.

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7. Endicott, K. M., and Yagoda, H., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 170.

8. Pele, S. R., *Nature*, 1947, v160, 749.

9. Boyd, G. A., and Williams, A. I., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 225.