TABLE II. Blood and Urine Levels of Patients Following I.V. Dextran.

Patient	Specimen	Blood mg %	Urine mg %	Total urine excretion, g
1	Control	14		
	Immed. after	715		
	24 hr	325	385	6.4
	48 "	121	149	3.4
	72 "	83	58	0.6
2	Control	10		
	Immed. after	682		
	24 hr	298	570	7.5
	48 "	222	495	5.9
	72 "	106	144	1.8

Table II records the blood and urine of 2 convalescent patients during the 72 hours following their intravenous injection with 500 ml of 6% dextran over a 30 minute period. These data are representative of a much larger series of observations made upon patients receiving dextran, and are intended to illustrate the useful application of the method to clinical studies involving the administration of dextran.

The method is not strictly specific, since it will measure the amounts of any polysaccharide resistant to digestion with hot alkali, precipitable by ethanol and capable of yielding a color with the anthrone reagent similar to or identical with that yielded by glucose. Substances meeting these requirements are not ordinarily found in significant amounts in blood and urine, so that measurements made after dextran administration may be taken with reasonable assurance that they reflect the dextran concentration in these fluids. The detection and measurement of dextran in tissue extracts containing glycogen, and possibly other polysaccharides, present another problem which is being attacked in experiments now in progress.

Received October 5, 1950. P.S.E.B.M., 1951, v76.

Radioautographic Solubility Studies of I^{131} and P^{32} in Frozen-Dehydrated Tissues. (18373)

MARGARET W. HOLT AND SHIELDS WARREN.

From the Laboratories of Pathology, New England Deaconess Hospital and Harvard Cancer Commission, Boston, Mass.

A previous report(1) described a method for preparation of radioautographs of animal tissues without isotope loss.* This work demonstrated the ease of extraction of P^{32} from frozen-dried liver sections when they come into contact with water. This result agrees with chemical studies of P^{32} distribution in tissues which have shown that a major part of the P^{32} should be present in water soluble form(2). The present report describes results obtained when frozen-dehydrated tissue sections containing radioisotopes are exposed to extraction by a variety of reagents, both aqueous and nonaqueous. Radioautographic exposures were made with the extracted tissue sections and also with non-extracted control sections, as will be described. The results were evaluated by comparing the photographic densities and pattern of localization of radioautographs obtained from analogous tissue sections subjected to various types of extraction with those of the control sections. It is an application of the radioautographic method to obtain information about the chemical nature of radioisotopes distributed in tissues.

Solubility studies have been of very limited value in conventional technic histo-chemistry as Cain(3) has pointed out in a recent critical discussion. Tissues prepared by quick-freezing and drying warrant further investigation

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

* With the possible exception of loss of lipoids into the paraffin used as the embedding medium. This point will be discussed later in the paper.

2. Hevesy, George, *Radioactive Indicators,* Interscience Publishers, Inc., New York, 1948, 344.

3. Cain, A. J., *Biol. Rev.,* 1950, v25, 73.

from this standpoint; however, certain limitations are inherent in all solubility studies(3). An advantage of the quick-freezing and freezing-drying process for preparing tissues consists of minimal alterations in chemical and biological characteristics of the system(4). Due to the porous solid framework that is formed, the solubility of compounds in such preparations is extremely rapid and complete, as was illustrated by Flosdorf(4) in the case of gelatin. When a thin (5 to 8 μ) frozen-dehydrated tissue section is treated with various solvents, the conditions for rapid extraction of the tissue should be optimal.

Tissues used in the present investigation were prepared and sectioned using the technics described previously(1).† The general procedure used in extracting the tissue sections is as follows. If the reagent being tested is not a paraffin solvent, the test is made by floating the coated section on the reagent with the cut surface down for five minutes. The section is then mounted on an albumin-coated cover glass which is placed in a vertical position in an oven at 48°C for an hour to allow firm attachment of the tissue section to the glass and to permit excess paraffin to drain off. The preparation is now ready for the radioautographic exposure or, alternatively, the paraffin may be removed from the section with xylol before making the exposure. We have not detected appreciable scattering or diffuseness of the radioautographic image due to the presence of a trace of paraffin. On the other hand, as will be pointed out later, xylol does not extract detectable amounts of I^{131} or P^{32} from the tissue sections that have been studied. If the reagent being tested is a paraffin solvent, the freshly cut tissue section is placed directly on the albumin coated cover glass (tissue side down), and the cover glass is placed in the oven as described previously. After removal from the oven, the preparation is immersed in the test solution; this removes the paraffin and subjects the tissue section to extraction simultaneously. The preparation is returned to the oven for a few minutes to aid in complete evaporation of the test solution before making the radioautographic exposure. The procedure used in making radioautographic exposures has been described previously(5).‡

Frozen dehydrated rat thyroid sections containing I^{131} were studied in this way. Numerous studies with conventional chemical and histological procedures have been made with this tissue and isotope. These have demonstrated that at very short time intervals (a few minutes) following the injection of I^{131}, over 90% of this isotope is present in the rat thyroid in protein bound form(6,7).

In the present investigation we used subcutaneous injection into rats of doses of carrier-free I^{131} ranging from approximately 0.5 μc/g body weight when given 4 hours before killing the animals to approximately 1.0 μc/g when given 72 hours before killing. A 3 hour radioautographic exposure of the frozen dehydrated thyroids gave satisfactory results 10 days after the injection. A large number of reagents, both organic and inorganic, were used; the solutions tested in general are those which are known to dissolve or precipitate iodine compounds (thyroxine, diiodotyrosine, and inorganic iodine) of interest in the thyroid and those which are known to cause co-

4. Flosdorf, Earl W., See Über, Fred M., *Biophysical Research Methods,* Interscience Publishers, Inc., New York, 1950, 213.

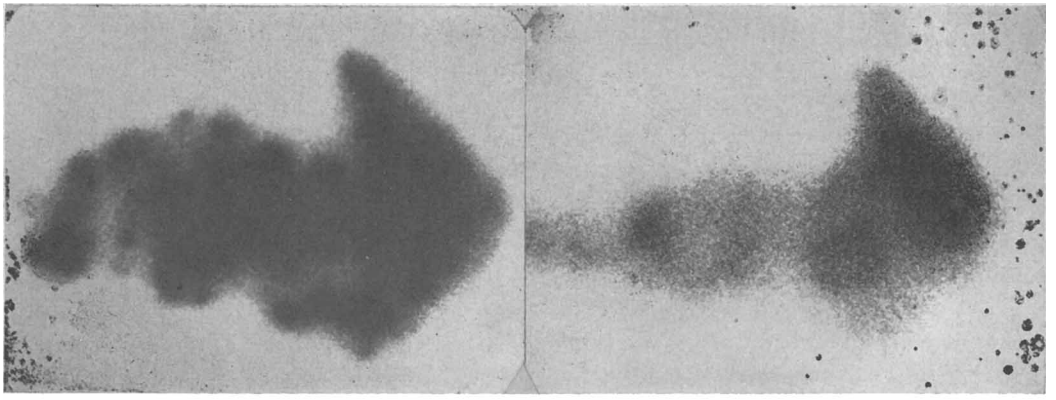
† We have found that, in cutting sections with this technique, the best results are obtained by painting the cut surface (by means of a camels hair brush) with paraffin at approximately 70°C. The paraffin is allowed to cool for about two seconds before cutting the section. (Sections between 2 and 8 μ in thickness are easily obtained).

5. Holt, M. W., and Warren, S., Soc. Proc. Exp. Biol. and Med., 1950, v73, 545.

‡ The plastic spacers used in the present work were 10 μ in thickness; they are obtained by immersing Eastman Kodak NTB stripping film in warm water to melt off the emulsion, then stripping the 10 μ film (from which the "spacers" were cut) from the cellulose support after it has been allowed to dry. In making the exposures, we have found it useful to place a microscope slide over the cover glass before the clamp is attached in order to prevent breaking the thin cover glass.

6. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.,* 1947, v169, 49.

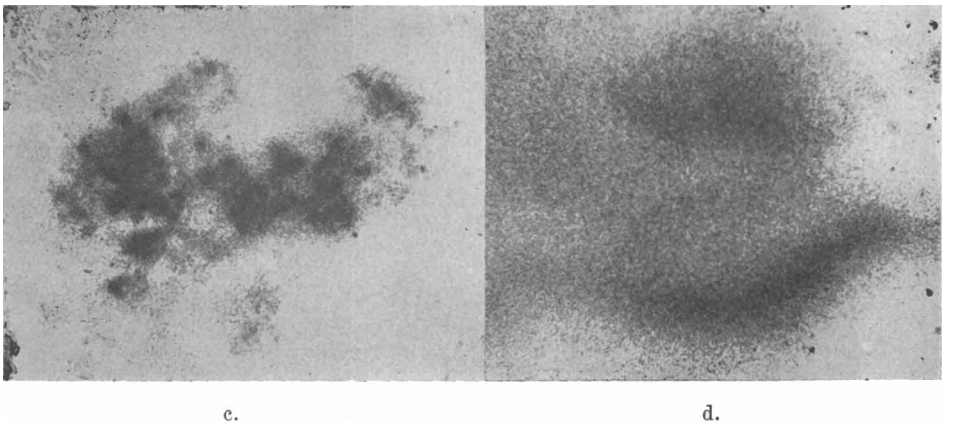
7. Leblond, C. P., and Gross, J., *Endocrinology,* 1948, v43, 306.



a. b.

FIG. 1a (left). Typical radioautograph of a section of frozen-dehydrated rat thyroid prepared without isotope loss. Areas of darkening are visible which represent localization of I^{131} in the follicles. This section was extracted with chloroform previous to the radioautographic exposure. ×50.

FIG. 1b (right). Radioautograph of an analogous section to that shown in *a* which was cut from the same tissue block. This section was extracted first with chloroform, then with distilled water before making the radioautographic exposure; comparison with *a* shows that the water dissolved out most of the isotope. ×50.



c. d.

FIG. 1c (left). Radioautograph of a thyroid section which is from a different block from those illustrated in *a* and *b*, but the thyroid tissue used for *c* was prepared prior to sectioning in the same way as the tissue used for *a* and *b*. This section was floated on saturated ammonium sulfate before it was mounted on the microscope cover glass. Xylol was used to remove the paraffin from the tissue. Comparison with *a* and *b* shows that some of the radioactive isotope was lost when the section was floated, but that the ammonium sulfate extracts considerably less of the isotope than distilled water does (the control sections on this block produced images very similar to that shown in *a*). This is typical of results observed with protein precipitants. ×50.

FIG. 1d (right). Radioautograph of a thyroid section which is analogous to those illustrated in *a* and *b*, but which was floated on distilled water before it was mounted on the microscope cover glass. Xylol was used to remove the paraffin from the tissue before making the exposure. Comparison with *a* shows that the isotope was partially washed from the tissue, resulting in a blurred image instead of the typical pattern of localization. Extraction with water usually resulted in almost complete loss of the image with the exposure times used. ×50.

agulation or denaturation of proteins. Fig. 1 (a, b, c, d) illustrates the type of results observed. Treatment with reagents which extract the radioactive material from the tissue either causes no image or a very light image to be formed on the radioautograph. In other cases, appreciable amounts of the radioactive material remained attached to the paraffin or cover glass surface, but smearing and blurring of the image showed that some

extraction of the isotope from its site in the tissue had occurred. The results were first observed grossly (*i.e.* density of the image) and in addition all of the images were examined microscopically for evidences of blurring or shifting in orientation of the radioautograph pattern. The radioautograph obtained from a section which had been mounted directly on the cover glass and subjected to no solvent extraction (merely to removal of all paraffin that could be melted off the glass) served as the control in evaluating the results.

No significant differences were noted in solubility studies made on thyroids of rats killed 2, 4, 20, 48 and 72 hours after injection of the I^{131}. The results obtained may be summarized as follows: (1) The image of the extracted section was indistinguishable from the control indicating that the radioisotope was insoluble in and not displaced by the reagent (Fig. 1a). This type of result was obtained with xylol, chloroform, anhydrous ether, absolute ethyl alcohol and n-butyl alcohol. The results also indicated the radioactive isotope was partially irreversibly precipitated by these reagents. This was demonstrated by subjecting the tissue to extraction with distilled water following treatment with the precipitating agent. The radioautographs obtained when treatment with water followed the precipitation showed that the isotope was less easily extracted by water as a result of the precipitation (Fig. 1b and 1d). (2) With protein precipitants such as 5% trichloroacetic acid, Bouin's solution, 95% ethyl alcohol, acetone, 1% ferric chloride, 5M sodium chloride, saturated ammonium sulfate, and strong mineral acids and bases (1N H_2SO_4 and in 1N NaOH), careful observations of the radioautographs showed that some loss of isotope occurred, but that a major fraction of the activity was retained in the tissue section (Fig. 1c). (3) Blurring of the image and/or a lighter image was observed, indicating that the radioisotope dissolved to an appreciable extent. For example, when the tissue section was treated with saturated KI solution, although a definite radioautograph image was visible, the major fraction of the activity had been extracted. This was obvious when it was compared with the radioautographs of control sections. In this case, the activity that was not extracted was apparently that present as inorganic I^{131}. (4) Essentially complete loss of the image, indicating that the radioisotope was soluble (Fig. 1d). This type of result was obtained with reagents which, in addition to dissolving inorganic forms of iodine, would be expected to dissolve native protein (or to denature the protein but not alter its solubility). These solutions included distilled water, 1M sodium chloride, 30% urea, 1% pepsin solution, and a detergent solution (sodium lauryl sulfate).

These solubility data correlate with those to be expected if a major portion of the radioisotope were incorporated into a water soluble protein molecule which is coagulated by the common protein precipitants. If the thyroxine were present in free form in the tissue, a detectable fraction (roughly 12-27%) of the radioactivity should be insoluble in water and soluble in alkalies and butyl alcohol. The interpretation that the I^{131} present in inorganic form is a relatively minor fraction of the total and that the thyroxine and diiodotyrosine are combined in the newly formed thyroglobulin molecule correlates with the chemical analyses quoted previously (6,7). The fact that water is less effective in dissolving the isotope following treatment with protein precipitants suggests a partially nonreversible. protein coagulation or fixation.

Similar studies were made on frozen-dehydrated rat tissue sections following subcutaneous injections of carrier-free P^{32} (approximately 5 μc/g). Results have been obtained at varying time intervals from injection of isotope to sacrifice of the animal. In general, the radioautographic exposure times were from 4 to 8 days using contrast lantern slides (exposures were started within one to 3 weeks following the injection).

The use of rather large doses of P^{32} was considered to be desirable in order to shorten the photographic exposure time. Studies of P^{32} distribution in tissues have indicated that with this particular isotope wide ranges of isotope dosages may be employed without altering the chemical distribution of the iso-

tope in the various tissue components(8).

Radioautographs of frozen-dehydrated rat liver sections demonstrated that the P^{32} is distributed uniformly in the tissue. Results will be given for a twenty-four hour time interval. Densities of radioautographs of sections extracted with various solutions were compared by means of an Ansco-Sweet densitometer. In general, comparable densities were observed for control sections (paraffin not removed) and for sections treated with chloroform, ether, xylol, absolute alcohol, and acetone. Treatment with distilled water, water solutions of pepsin, urea, dilute sulfuric acid, half-saturated ammonium sulfate, and Bouin's solution decreased the density of the image to approximately one-fourth that observed for control sections (this was only slightly above background), indicating that the isotope dissolved in all of these solutions. Treatment with ether, chloroform, or acetone followed by treatment with water similarly resulted in loss of activity. Sections treated with an inorganic phosphate precipitant (slightly alkaline magnesium carbonate and calcium chloride mixture) retained a major fraction of the radioisotope, and similar results were obtained with sections treated with saturated Na_2HPO_4.

These results likewise correlate with chemical analyses(9) in showing that the radioactive material is present in water soluble and acid soluble form. The lipoid solvents do not remove detectable amounts of activity. This suggests that the lipoids were either extracted by the paraffin used for infiltration of the tissue or dispersed in the water solutions used as reagents, perhaps in colloidal form. The protein precipitants do not retain appreciable amounts of the activity in the tissue.

Other rat tissues containing P^{32} have been studied in a similar manner. These include the duodenum, small intestine, kidney, spleen, and Walker rat carcinoma. The time intervals varied between two and forty-eight hours from injection of isotope to killing of animal. With the exception of the Walker carcinoma, each of these tissues exhibits a nonhomogeneous isotope distribution. In general, the results observed are roughly comparable to those which have been described for rat liver. Images indistinguishable from those of the controls were obtained with organic solvents in general (xylol, ether, chloroform, acetone, absolute alcohol). Definite images were usually obtained with sections treated with solutions designed to retain $PO_4^{\equiv}$ (saturated Na_2HPO_4 or the phosphate reagent mentioned above) and with sections treated with protein precipitants (half-saturated ammonium sulfate, Bouin's solution). Distilled water and dilute water solutions dissolved enough of the isotope so that no image was visible when these extracted tissues were exposed for a period of time sufficient to give a satisfactory radioautograph of the control section.

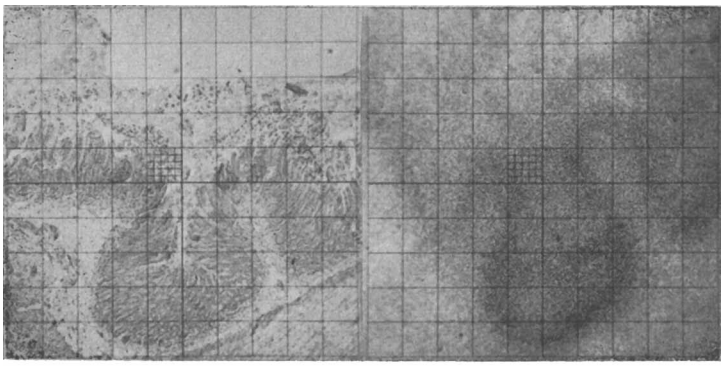
The regions of selective deposition of the isotope that have been studied exhibited the same solubility characteristics as the background activity in the tissue (Fig. 2).

The possibility of the loss of lipoid material into paraffin during the infiltration of frozen-dried tissues needs further investigation. It would be desirable to use other embedding media (with solubility characteristics different from that of paraffin) for purposes of comparison.

The results which have been reported are believed to be of value in giving more information regarding the methods to be used in handling frozen-dried material with isotopes such as P^{32} and I^{131} in order to prevent isotope loss. They also apply to the handling of frozen-dried material in general, when it is desirable to avoid loss of tissue constituents. In most cases, the results agree well with data obtained from chemical analyses of similar tissues, indicating that it may be possible to utilize these methods to obtain information about the chemical nature of isotope distribution. With tissues which exhibit localized concentration of the isotope such as the glands of the duodenum and small intestine which concentrate P^{32} (Fig. 2), it is particularly

8. Lawrence, John H., and Hamilton, Joseph G., *Advances in Biological and Medical Physics*, Academic Press, N. Y., 1948, v1, 324.

9. McCarter, J. A., and Stiljes, E. L., *Can. J. Res.*, 1948, v26E, 344.



a. b.

FIG. 2a (left). 5μ section of small intestine of 160 g rat prepared by quick freezing and dehydration and paraffin embedding. Animal sacrificed 4 hr after administration of 590 μc carrier-free P^{32}. Hematoxylin stain. ×50.

FIG. 2b (right). Radioautograph of a 5μ section cut from same block as tissue section shown in *a*. A 10 μ plastic ring spacer was used to separate the tissue section from the emulsion of a contrast lantern slide during the 11 day radioautographic exposure (exposure was started 10 days following sacrifice of animal). Xylol was used to extract the paraffin from the section previous to the exposure. ×50.

interesting to compare the properties of the localized isotope with those of the background activity in the tissue. It is possible that if the localized P^{32} is different chemically, further tests designed for specific differential precipitation might give information about the nature of the compounds present in a given location. Our results have shown that inorganic phosphorus and proteins can be precipitated *in situ* in the tissue by use of the proper reagents. After the tissue section is mounted, treatment with the proper fixative should not cause loss of the test material (the results have shown that acetone, for example, could be applied without loss of I^{131} or P^{32}); however, the effectiveness of the fixative is questionable since the results have shown that precipitation by such reagents is only partially irreversible. In testing for a given material in frozen-dried tissue sections, the procedure to be followed should be (1) Cut and mount the tissue section without contact with any reagents; (2) Remove the embedding medium with a solvent in which the test material is insoluble; (3) Apply a reagent designed toward specific differential precipitation of the test material, and (4) Employ other procedures (specific stains or radioautographic exposures) which make it possible to visualize the test material.

One of us (M.W.H.) wishes to express appreciation to the Atomic Energy Commission for a National Research Council A.E.C. Postdoctoral Fellowship in the Biological Sciences, which made this research possible. A portion of this work was done under Atomic Energy Commission contract number AT (30-1)-901 with the New England Deaconess Hospital. The isotopes used were supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn. We also wish to express our appreciation to Miss Alice A. Williams for technical assistance in part of this work.

Received October 23, 1950. P.S.E.B.M., 1951, v76.