AUTORADIOGRAPHIC LOCALIZATION IN THE TISSUES OF DRUGS AND METABOLITES

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LIKE histochemistry and fluorescence microscopy, autoradiography aims at localizing chemical compounds in histological sections.

If comparisons are made between these methods it appears that a disadvantage of autoradiography is that the possibilities for making detailed observations are not as good as with the others.

In histochemistry and fluorescence microscopy the observations are made directly on the sections. But in autoradiography they are made on a photographic emulsion at some distance from the radiation source in the section, and the spread of the radiation limits the resolution.

An advantage with autoradiography on the other hand — compared with the other methods mentioned — is that there is no difficulty in distinguishing an injected substance from substances which are normally found in the body. It is thus easier to follow the absorption, distribution and excretion pattern of an injected substance.

Autoradiography affords the possibility of beginning with a survey of the general distribution and then gradually concentrating on details, as illustrated in Fig. 1 a—d from an investigation by E. Hansson[1] concerning the formation of pancreatic juice proteins. The series of autoradiograms illustrates the distribution of ^{35}S-methionine first in the whole animal and then stepwise down to the cellular level.

A limitation in the use of autoradiography to study substances which are metabolized in the body is that only the distribution of the activity is followed. One cannot in the autoradiogram distinguish the unaltered substance from conjugated forms or from labelled fragments of the molecule. On the other hand the tracer technique offers very promising possibilities in detailed studies of metabolism if autoradiography is combined with micro-separation methods. The potentialities of this combination are especially good if a substance is available in several labelled forms with each form labelled at a particular site of the molecule.

ISOTOPES AND LABELLING

An autoradiographic investigation requires access to appropriate radioisotopes or labelled compounds.

The type of radiation which is generally used in biological autoradiography is β-radiation and the isotopes used most frequently are ^{14}C, ^{3}H, ^{35}S, ^{32}P and ^{131}I.

A rapidly increasing number of labelled metabolites containing these or other isotopes are now becoming commercially available. Work with drugs, however, generally presents the investigator with the problem of labelling the substance himself or arranging to have this done, which may involve high costs.

The development of methods for ^{3}H-labelling by catalytic hydrogenaction[2,3,4] and particularly by gas exposure[5,6,7,8,9,10] has greatly increased the possibilities for preparation of labelled drugs. Previously a radioisotope had to be incorporated during the course of a chemical synthesis or a biosynthesis while the tritium methods make it possible to label a commercially available nonradioactive drug.

The gas exposure technique also has the advantage that it is applicable to the labelling of a great variety of substances. It is, however, often difficult to obtain a radiochemically pure substance. The specific activity is only moderate and the activity is randomly distributed throughout the molecule.

Catalytic hydrogenation gives more defined localization of the label and often a higher specific activity but its applicability is more limited. A way of increasing the applicability of catalytic hydrogenation is to synthesize unsaturated intermediates for subsequent tritiation or to tritiate a halogenated intermediate.

Autoradiographic Technique

Since most drugs are in a water-soluble form in the tissues, customary histological techniques such as chemical fixation and decalcification cannot as a rule be used.

In our laboratory an autoradiographic technique has been developed which is well suited for work with water-soluble substances, and which has been used in a great number of drug distribution studies.

It allows thin sections to be taken through whole small experimental animals such as mice and rats.

A number of small experimental animals, usually mice, are injected with the labelled substance and at different intervals after the injection they are rapidly frozen by immersion in a cold liquid at the temperature of solid carbon dioxide or liquid nitrogen and transferred to a refrigerated room kept at $-10°C$ or an open freezer at $-20°C$.

Sagittal sections through the animal are cut at different levels. To obtain whole sections an adhesive tape (Scotch cellulose tape) is applied to the section surface of the frozen specimen before cutting (Fig. 2) and the section then adheres to the tape. The section thickness is generally 20 μ but 5 μ-sections and occasionally whole 2 μ-sections may be taken.

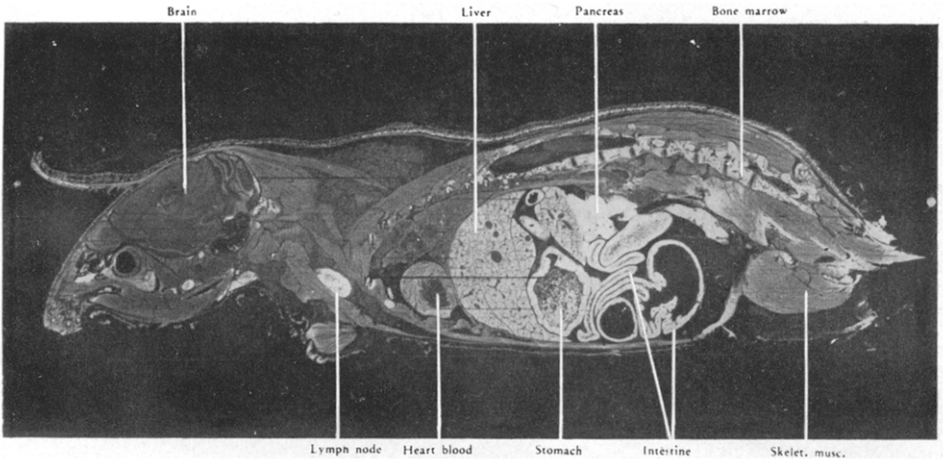

FIG. 1 a. Autoradiogram showing the distribution of radioactivity in a mouse 30 min after intravenous injection of ^{35}S-methionine. The figure shows only the autoradiogram. The section is removed. White areas in the autoradiogram correspond to high radioactivity. The highest uptake is seen in tissues with a rapid enzyme synthesis such as pancreas and gastrointestinal mucosa, and cell synthesis such as the bone marrow and lymph nodes. Section thickness: 20μ.

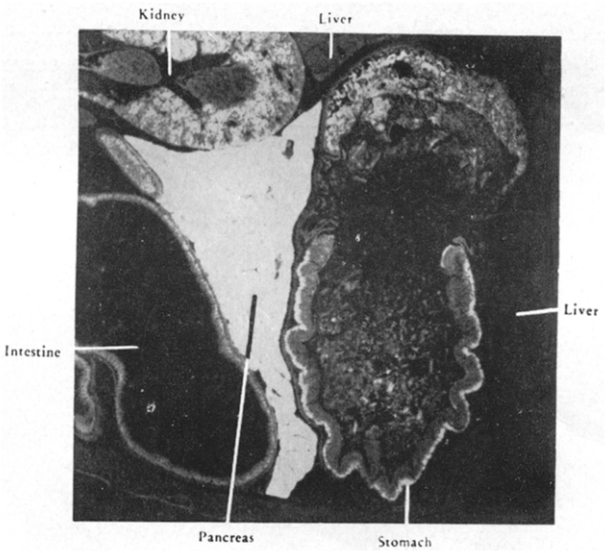

FIG. 1 b. Detail of a whole body autoradiogram from same animal as Fig. 1. Note high activity in pancreas, zymogen cells of gastric mucosa, intestinal mucosa and kidney.

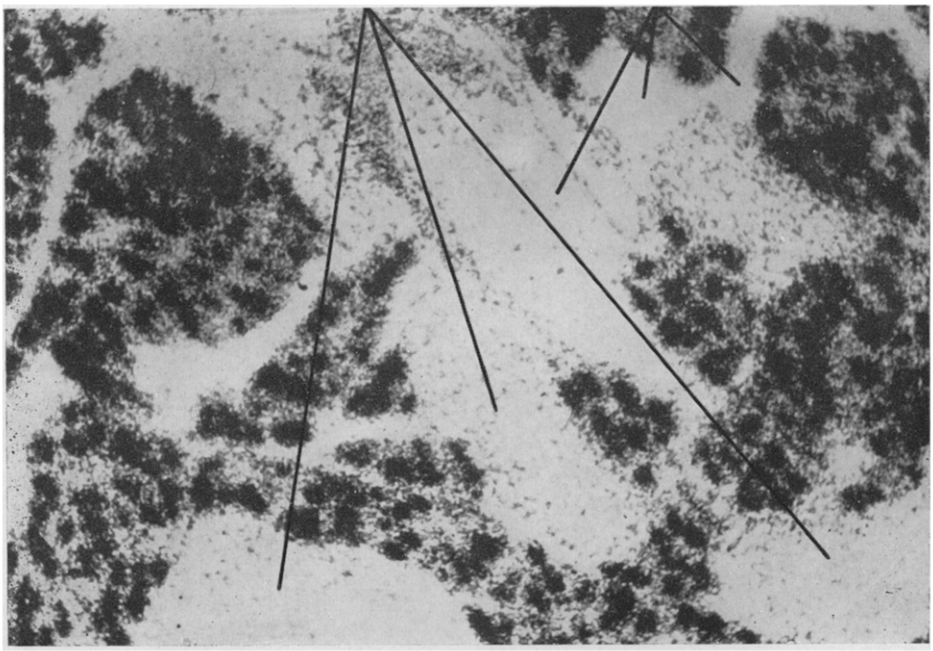

FIG. 1 c. Autoradiogram, 5μ section of pancreas from same investigation 60 min after injection of ^{35}S-methionine. The accumulation of black grains here indicates deposition of radioactivity. Stripping film autoradiography was used. High activity is in the exocrine glands but low in the connective tissue and in the islands of Langerhans.

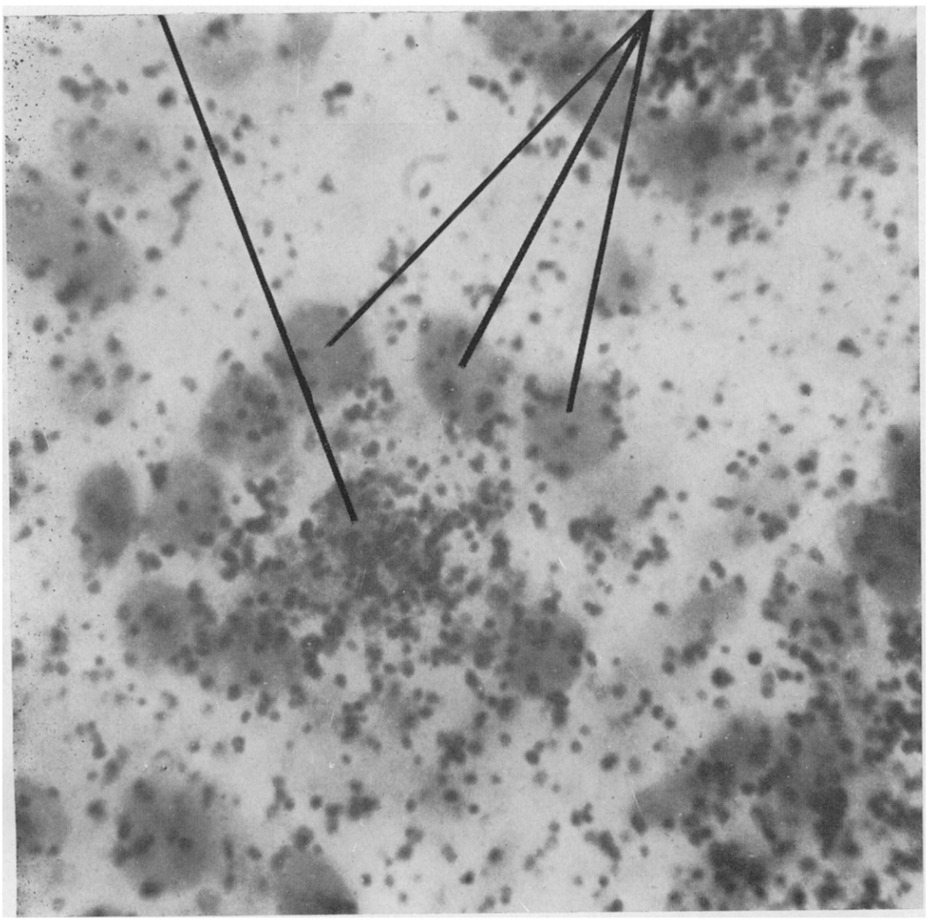

FIG. 1 d. A still more detailed picture from a stripping film autoradiogram of pancreas 1 hr after injection, showing a pancreatic acinus. The highest activity is seen in the lumen of the acinus indicating that the injected methionine is to a great extent in zy-

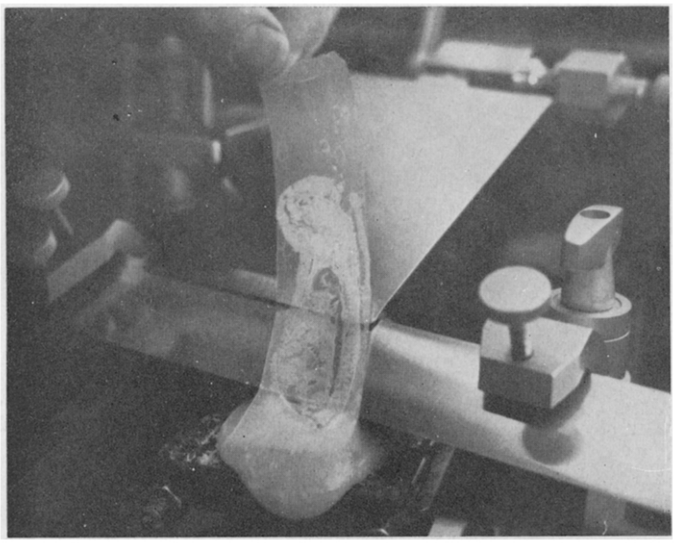

FIG. 2. Sectioning of frozen adult mouse at −10°C. Sagittal sections are taken at different levels. Sectioning is made through undecalcified hard tissues. The sections are fastened to tape. The section thickness is generally 20 μ occasionally 5 μ. Microtome: Leitz Grundschlitten.

Fig. 3 shows the sectioning of the head of a 3-year-old Rhesus monkey. A heavy microtome (Jung, type K) is used for sectioning very hard specimens.

The whole-animal sections are dried at −10°C and autoradiographic exposure is made at the same temperature. The sections may then be stained without removal from the tape. Fig. 4 shows a section through a pregnant mouse which is stained and mounted in euparal.

By using this technique artefacts affecting postmortem distribution and metabolism are avoided. Not only selected organs but practically all tissues and body fluids can be studied and this will often afford valuable information in addition to that which is sought. The technique also permits short-interval studies to be performed (e.g. 1 min after intravenous administration).

Detailed Autoradiography

For apposition autoradiography the histological section and the film are pressed together during exposure and are then treated separately.

Details can more readily be studied if small sections are permanently combined with a thin and fine-grained photographic emulsion. Both the section and the attached emulsion are then passed together through photographic processing and staining. The distribution pattern can be observed by light microscopy at high magnification by focusing alternately on the histological section and the developed grains in the emulsion (Fig. 5).

Small areas cut out from thin whole-body "tape sections" may be used. Other possibilities for the study of water-soluble isotopes are to section small fresh ice-mounted tissue specimens[14] (frozen at as low a temperature as possible to avoid ice crystal formation) or to use freeze-dried and paraffin-embedded specimens[7,15]. The sections are dry-mounted on appropriate autoradiographic plates.

When the isotope is in a firmly bound form in the tissues, small specimens can be removed and fixed chemically and autoradiographic methods such as wet stripping, immersion in liquid emulsion, wet mounting or painting of the sections with emulsion may be used.

Photographic Emulsions

Of the commercially-available films, coarse-grained röntgen films are the most sensitive for β-radiation (except for tritium).

Great progress in high resolution autoradiography was made when the nuclear emulsions — developed by the nuclear physicists — could be adapted for autoradiography. These emulsions are characterized by a small and uniform grain size and high grain density.

Another contribution has been the development of special emulsion forms like stripping films (which can be stripped off from the supporting plate).

Resolution

The resolution in autoradiography mainly depends on the thickness of the section, the emulsion and the layer between them.

The resolution also depends on the energy of the isotope. For tritium with an average range in the emulsion of less than 1 μ the resolution will be significantly better than with other β-emitters (Fig. 6).

It is also easier to obtain good resolution with a substance which accumulates strongly in defined structures than with a substance which is more evenly distributed, and it is easier to work with a substance which is firmly bound than with a water-soluble substance. Optimal conditions for obtaining good resolution are afforded by ^{3}H-thymidine (Fig. 7).

Recently, attempts have been made to obtain better resolution by electronmicroscopic examination of autoradiograms from ultra-thin sections[16,17,18].

Sensitivity

Autoradiography is a relatively insensitive method if the radioactivity doses that are needed are compared with the doses which are required for impulse counting of excised organ pieces. But the method is sensitive in the sense that the activity in fine structures can be recorded.

The sensitivity is highly dependent upon the specific activity of the radioactive substance used. If a substance of high specific activity is used, an investigation can be performed with a relatively small dose. The specific activity of an isotope depends upon whether the isotope can be produced by a transmutation reaction as "carrier free" or whether a certain amount of carrier (the inactive atom) cannot be avoided. The half-life of the isotope also plays a role.

The specific activity of an organic compound also depends on the method of labelling. For economical reasons a high specific activity generally requires that labelling be performed on a micro scale.

Quantitative Autoradiography

Quantitative estimation on "contrast autoradiograms" may be made by densitometry. A good reference in such measurements is a staircase containing the particular isotope in uniform concentration steps which is placed beside the section during autoradiographic exposure.

When preparing an isotope staircase the gelatin layer of a single-coated photographic film is a suitable absorbent for the isotope.

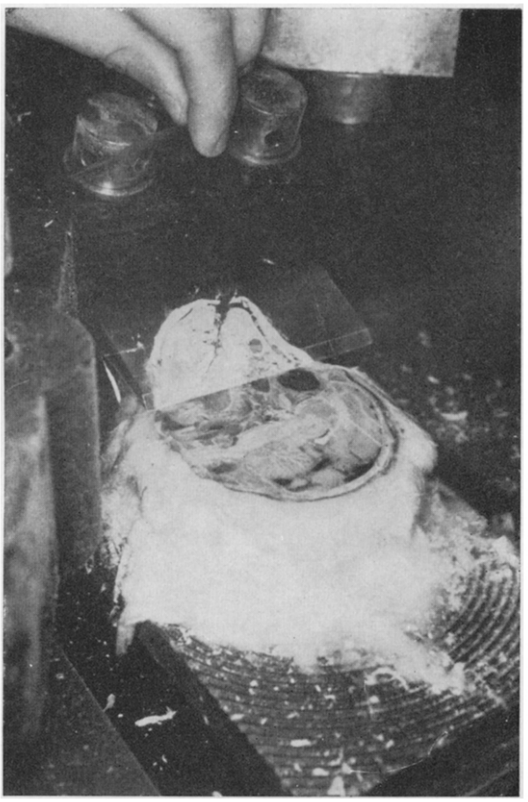

FIG. 3. Sectioning of the head of a 3 year old Rhesus monkey. The microtome knife has just passed through the teeth of the monkey. Microtome: Jung, type K.

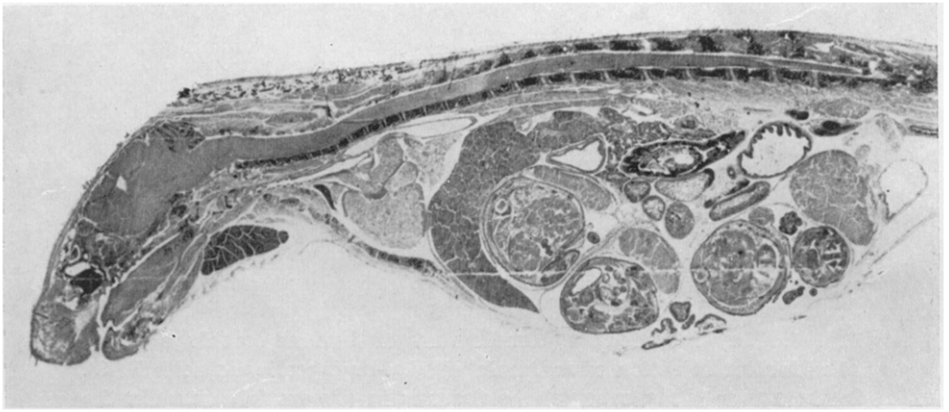

FIG. 4. A 10μ thick section through a pregnant mouse. The section is stained with haemalun-eosin and mounted in euparal.

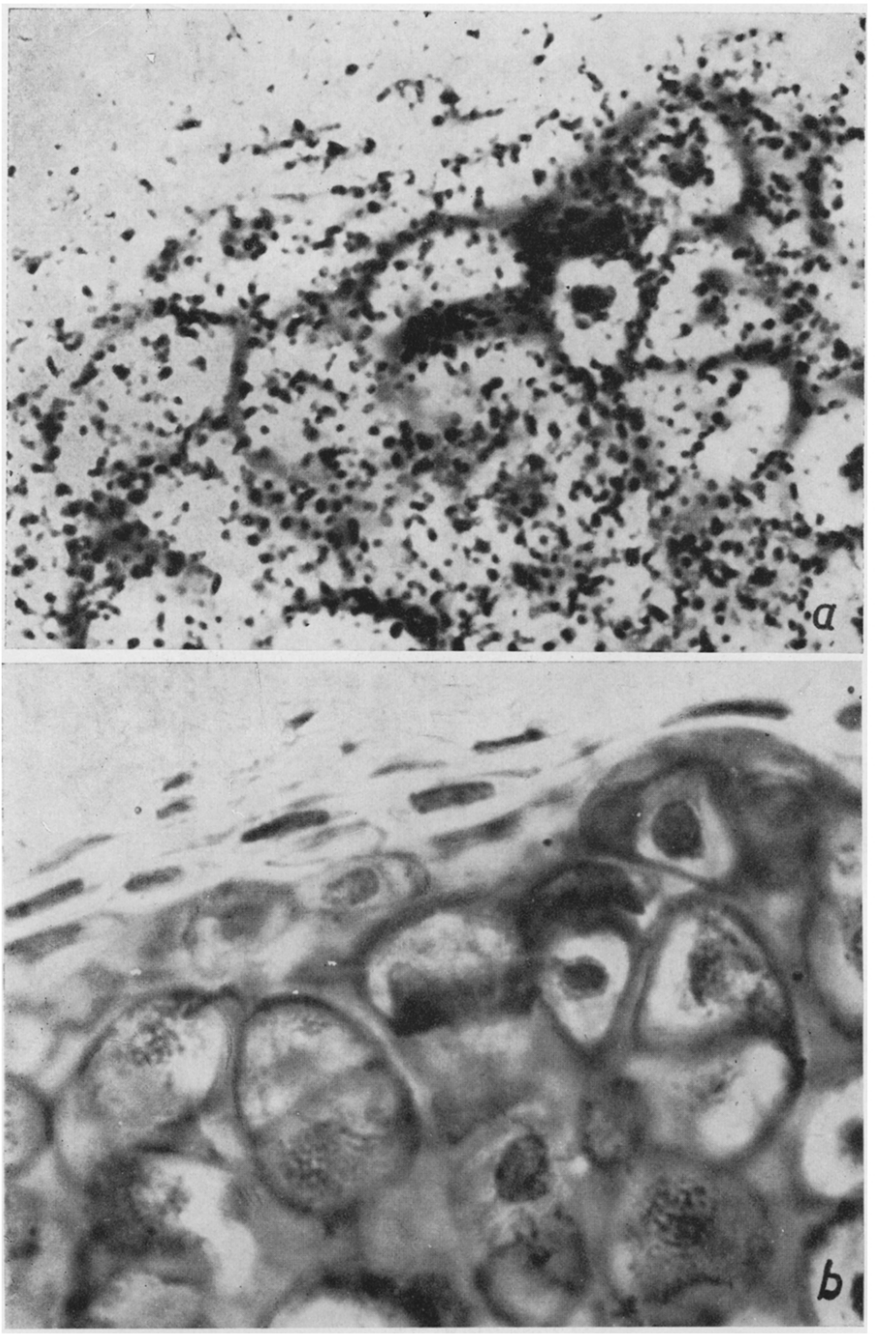

FIG. 5. "Dry-mounted" autoradiogram of hyaline cartilage tissue 30 min after injection of tritiumlabelled p-aminosalicylic acid (PAS). In the upper photograph (a) the focus is in the emulsion and in the lower (b) in the section. Note radioactivity in cartilage capsules and in cells at the border between cartilage and perichondrium. E. K. Stripping film. × 1800.

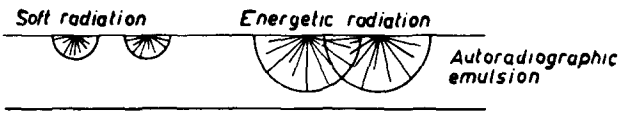

FIG. 6. The influence of the energy of the isotope on the resolution: A weak beta emitter may produce two distinctly separable blackenings of the emulsion (left) while a harder radiation produces a diffuse blackening.

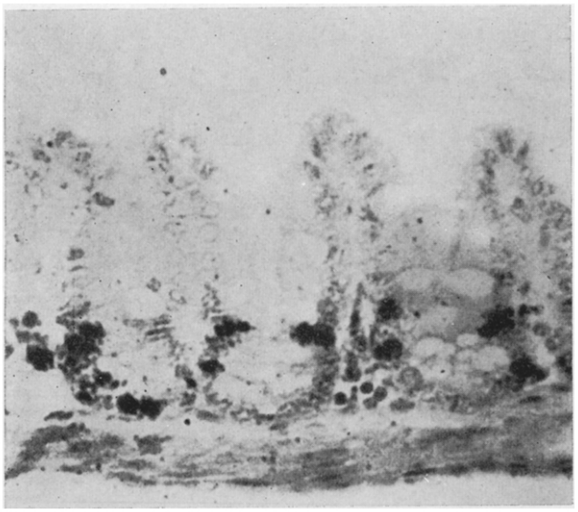

FIG. 7. The DNA — precursor thymidine — ^{3}H-labelled — is taken up by dividing cells. Autoradiogram from mucosa of colon 8 hr following injection. A number of strongly labelled nuclei are visible in the lower part of the colonic crypts. — From Leblond *et al. Lab. Invest. 8* 269 (1959).

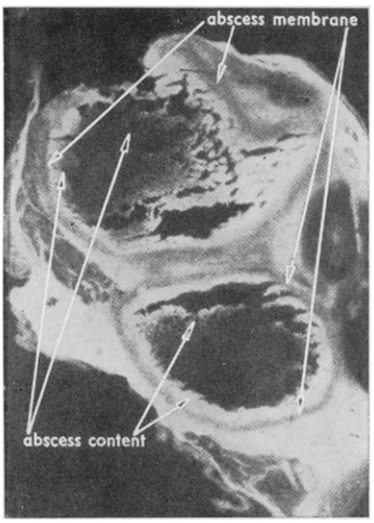

FIG. 8. Autoradiogram showing the distribution picture in two neighbouring 30 days old abscesses 30 min after injection of 35S-penicillin. Note high content of radioactivity immediately inside the abscess wall and falling concentration towards the centre

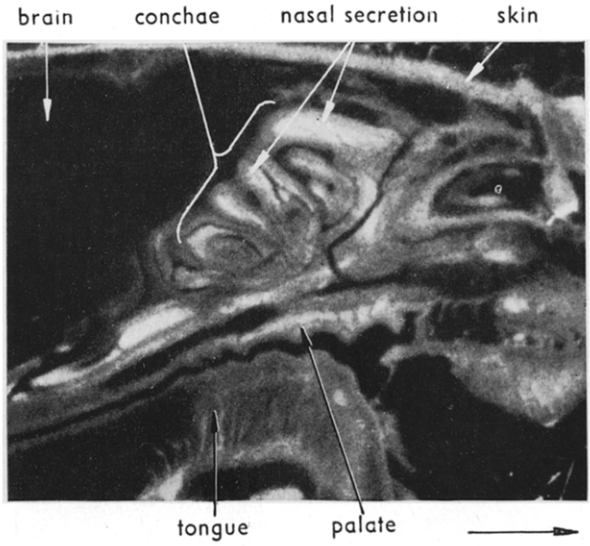

FIG. 9. Autoradiogram showing high concentration in the nasal secretion 30 min after intravenous injection of ^{35}S-penicillin.

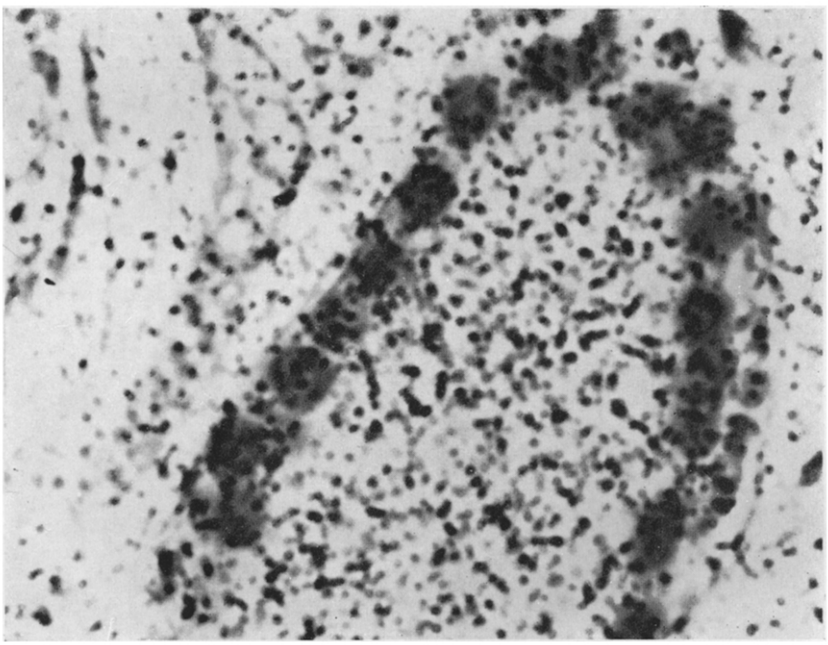

FIG. 10. Dry-mounted autoradiogram, section of the tryroid gland 30 min after injection of ^{3}H-para-aminosalicylic acid (PAS). Note high activity in the epithelium and follicle. E. K. Stripping film.

Quantitative results can also be obtained by Geiger-Müller or liquid scintillation counting of small pieces punched from dried large "tape sections".

In microautoradiography, grain counting is a reliable quantitative method although tedious. Equipment for automatic grain counting has been developed[19].

TYPES OF PROBLEMS

A fairly extensive review of the autoradiographic literature in the field of pharmacology has recently been given[13].

Most of the observations made up to now have been made on the tissue level rather than on the cellular level and the autoradiograms shown will therefore mainly be survey pictures.

The approach to the problems naturally varies from study to study and is determined among other things by the particular substance which is of interest.

The therapeutic effect of *antibiotics and chemotherapeutic agents* depends upon their ability to reach the invading micro-organisms. Autoradiography with antimicrobial agents may give information concerning the ability of these drugs to reach the commonest sites of infection, and their capacity to pass physiological barriers and such pathological barriers as a wall of an abscess. Fig. 3 illustrates the ability of ^{35}S-penicillin to cross an abscess membrane[11].

The different routes of excretion also appear on the autoradiograms. Fig. 9 shows one of the minor pathways for penicillin excretion which seem to be of therapeutic significance: excretion through the nasal mucous membrane.

An autoradiographic investigation with an antimicrobial agent can also give information concerning its toxic action. An investigation by Hanngren[7] with the antitubercular agent para-aminosalicylic acid (PAS) has shown that the PAS accumulates selectively in the thyroid (Fig. 6). This may be related to a finding by the same author that PAS blocks the radioiodine uptake by the thyroid and if used extensively has a goitrogenic effect.

Accumulation and retention of a *toxic substance* may be compared with morphological changes or with the clinical picture. For substances which are suspected of having a cumulative toxic effect autoradiography may reveal a potential risk.

The transfer of a drug across the placenta is often found to be blocked. For ^{58}Co-vitamin B_{12}, however, we have seen the opposite — signs of an *active transportation*[20] through the placenta. Fig. 11 shows a very rapid and pronounced accumulation of vitamin B_{12} in the placenta, which after a latent period of some hours is followed by a marked accumulation in the foetus. The foetal tissues gradually attained a much

higher concentration than the maternal. The observations demonstrate a rate of transfer similar to that for absorption of vitamin B_{12} across the intestinal mucosa[21], and it does not seem unlikely that the transportation in the placenta, as in the small intestine, is controlled by the intrinsic factor or a similar substance.

In attempting to relate specific accumulation of a drug to its *mode of action* it is often difficult to judge whether the uptake is associated with effect or whether it simply reflects storage. This is a problem shared with histochemistry.

When accumulation is observed in a receptor with a known function the results can be interpreted more precisely. For example, Waser and Lüthi have found that substances with a curare-like effect are selectively localized in the muscle end-plate region in the diaphragm[22,23]. They obtained elegant results by making autoradiograms from whole air-dried diaphragms of mice (Fig. 12). Grain counts of their autoradiograms from thin sections also indicated an accumulation. Later investigations[24] showed that neurotomy of the phrenic nerve slowly decreased the accumulation in the end-plate region of ^{14}C-curarine and that the administration of a potentiating agent (SKF 525 A) increased the accumulation.

In connexion with the study of endogenously-formed substances of pharmacological interest one approach might be to investigate the fate of its presumed precursors. After injections of the amino acid analogue ^{14}C-dihydroxy-phenylalanine (dopa)[25] the distribution picture during the first hour has been very similar to the one which has been obtained with the "protein-building" amino acids tyrosine and phenylalanine. Accumulation was noticed especially in organs with a rapid protein synthesis such as the pancreas (Fig. 13). An accumulation in the adrenal medulla was also seen. Later on, the radioactivity disappeared from most organs but increased in the adrenal medulla. The autoradiograms 4 days after injection showed ^{14}C exclusively in the adrenal medulla.

A few investigations have shown how a difference in effect between two substances with a *related chemical structure*[26,27] can be paralleled by a difference in distribution pattern as seen on autoradiograms.

The intriguing relationship between chemical structure, distribution, and effect is one in which the autoradiographic distribution pattern may serve as a guide. Autoradiography will undoubtedly find many applications in studies of these aspects.

Technical Possibilities

The useful range of autoradiography can be widened by different technical approaches. One of these is to combine autoradiography with *selective extraction* from the section of a component of the labelled compound. The procedure has been used with apparently good results

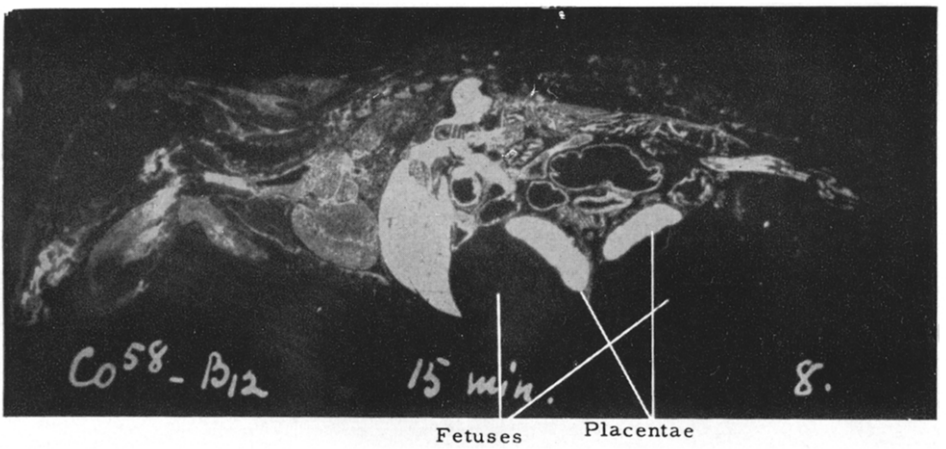

FIG. 11 a. Autoradiogram showing distribution of ^{58}Co-vitamin B_{12} in a pregnant mouse 15 min after intravenous injection. White areas correspond to high radioactivity. Note the heavy accumulation in the placentae. No radioactivity is discernible in the foetuses.

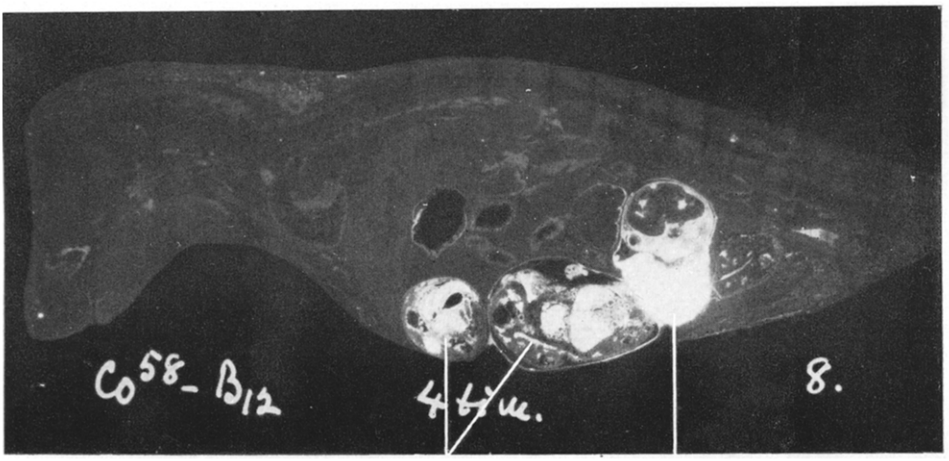

FIG. 11 b. Autoradiogram of ^{58}C-vitamin B_{12} in a pregnant mouse 4 hr after intravenous injection. A very heavy accumulation of radioactivity in the foetuses compared with the mother can now be seen. All foetal tissues show higher activity than the corresponding maternal tissues. The concentration in the placentae is still high.

FIG. 12 a. Photograph of a mouse diaphragm with specifically stained end plates.

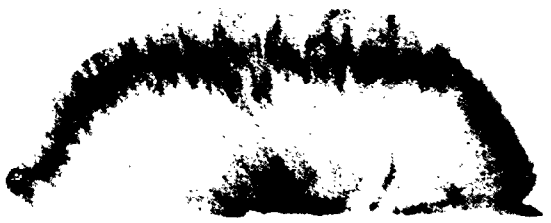

FIG. 12 b. Autoradiogram of the same diaphragm showing accumulation of ^{14}C-decamethonium in a region corresponding to the end plates. From P. Waser, M. Lüthi: *Nature, Lond.* 178 981 (1956).

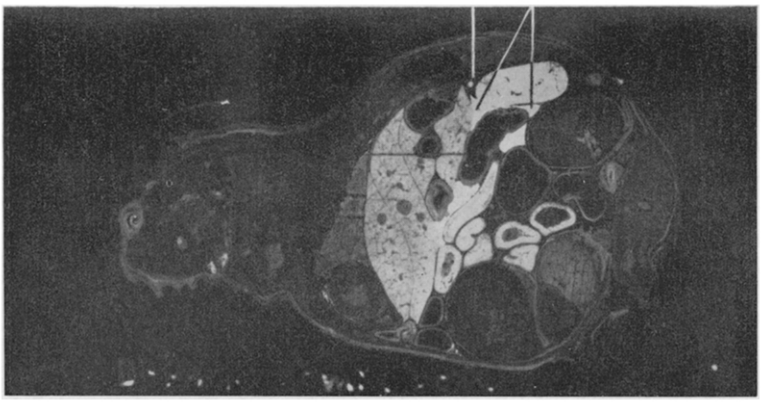

FIG. 13 a. Autoradiogram showing the distribution of ^{14}C-dihydroxyphenylalanine (dopa) 30 min after intravenous injection. The distribution is now similar to that found with the "ordinary" amino acid phenylalanine. High uptake in pancreas, intestinal mucosa, kidney and liver. Accumulation is beginning in adrenal medulla.

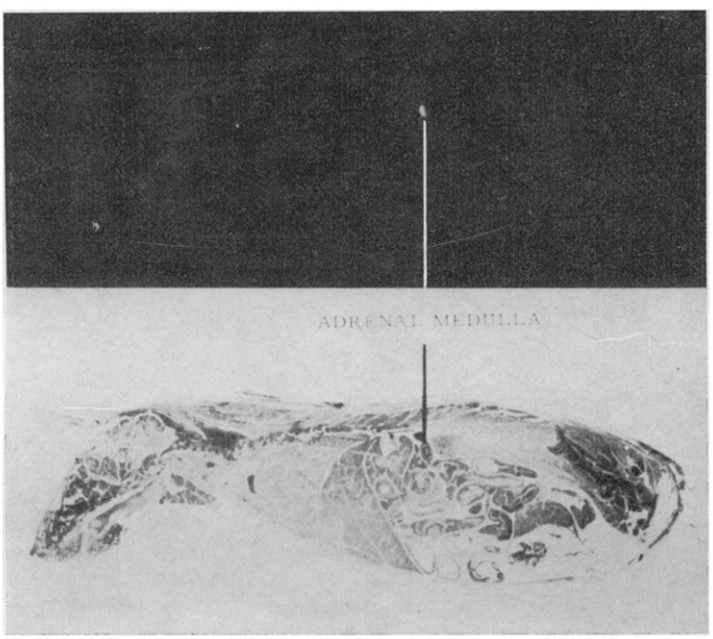

FIG. 13 b. Autoradiogram (above) and photograph of corresponding stained section (below) from a mouse 4 days after injection of ^{14}C-dopa. Radioactivity can now be observed exclusively in the adrenal medulla.

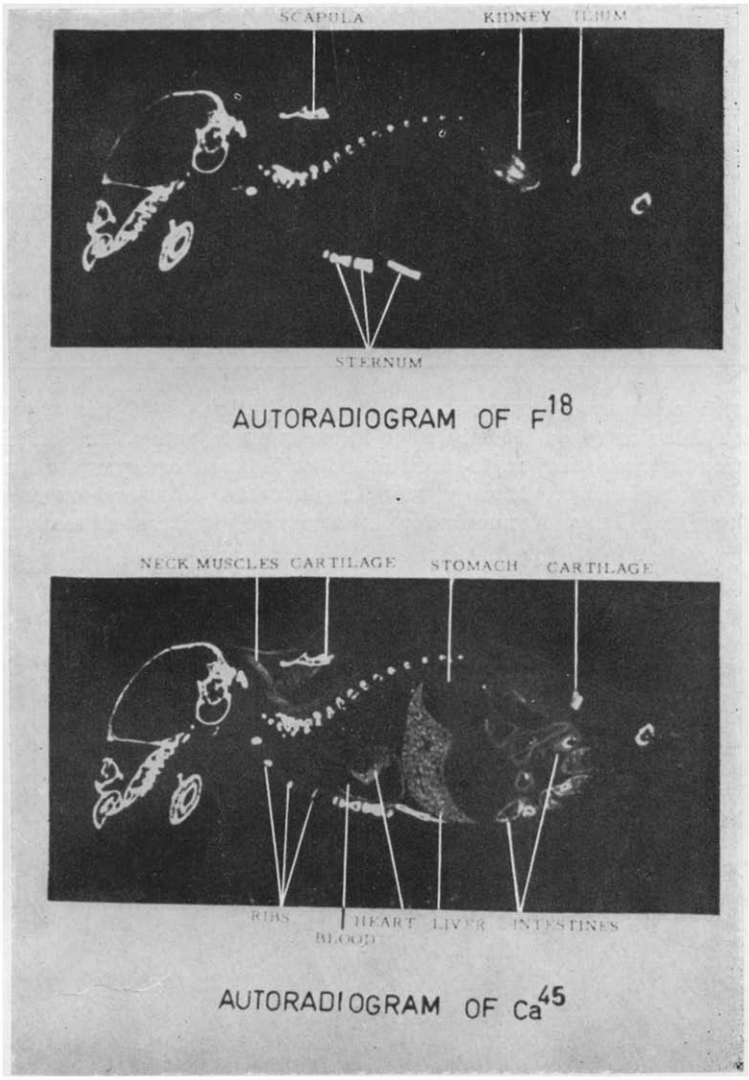

FIG. 14. Double isotope autoradiography: A comparison between the distribution of radiofluorine and radiocalcium in the same section 30 min after the simultaneous intravenous injection of the two isotopes. The autoradiogram above shows the distribution of ^{18}F, which has accumulated selectively in the hard tissues. Accumulation of ^{45}Ca (below) is seen in the hard tissues but also in some muscles and in the liver and small intestine.

in an investigation with labelled amino acids[28]. Sections through animals which had been killed at intervals after an intravenous injection of labelled amino acids were autoradiographed in the usual way. Then, after treatment of the sections with a trichloroacetic acid solution (TCA) to remove free amino acids, exposure was repeated. In this manner, the autoradiogram from the sections not treated with TCA illustrated tissue accumulation of amino acids from the blood, a process which was nearly complete after a few minutes. The TCA-treated sections and their autoradiograms revealed the rate at which the amino acids were incorporated into tissue components, particularly proteins, a much slower process than the mere accumulation of amino acids.

Another technical possibility is to study the distribution of two isotopes simultaneously in the same section and in this way compare their distribution patterns, without disturbing influence from biological variation[29]. Fig. 14 shows a comparison between the distribution of fluorine and calcium. Fluorine-18 has a half-life of less than 2 hr. The two isotopes were given simultaneously and the short-lived fluorine dominated totally, initially, while the radio-calcium was registered after the decay of radio-fluorine.

It has also been possible to obtain a detailed localization of ^{18}F in developing teeth of a young rat (Fig. 15) in spite of its short half-life[14]. By use of rapid autoradiographic techniques it is thus possible to work with short lived isotopes if the investigations are carried out in the neighbourhood of an atomic reactor or cyclotron.

New Pathways

Further developments in autoradiography will undoubtedly arise from the aspects discussed here as well as others which have as yet scarcely been contemplated.

We can expect a much greater number of suitable isotopes to become available, in part because the supply of suitable targets can be increased, as more stable isotopes are produced by mass spectrometry.

Labelling techniques are also developing rapidly and we probably soon shall be able to obtain commercially labelled compounds with a greater specific activity and which offer a wide range of choices in the nature of labelling and the position in the molecule occupied by the active isotope.

As for resolution, electron microscopy in combination with autoradiography has already been attempted with promising results. When the technical difficulties are overcome, then great strides will no doubt be made toward better resolution. Another possibility, as yet theoretical, is to collimate and possibly focus the radiation. Isotopes which emit monoenergic electrons such as internal conversion electrons would be most suitable for such a technique.

Technical developments, of course, are only one aspect. Newer applications of autoradiography will undoubtedly keep pace with these.

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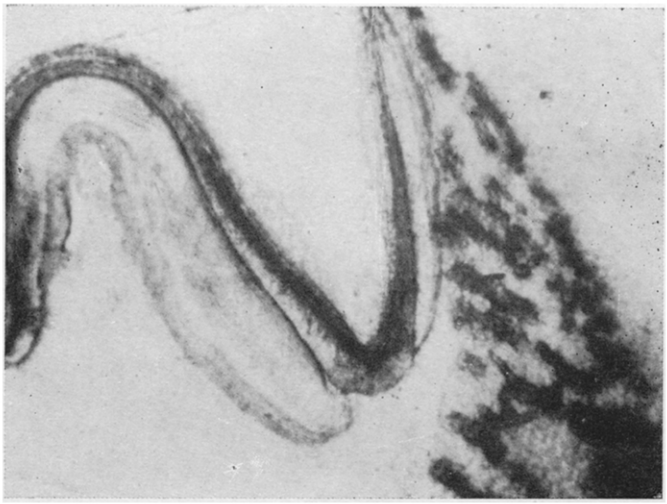

FIG 15. "Dry-mounted" section and autoradiogram illustrating the possibilities of using short-lived isotopes in microautoradiography. Radiofluorine (^{18}F) with a half-life of 110 min is incorporated in dentine, enamel and alveolar bone of a 15 day old rat.

DISCUSSION

Dr. STEVEN E. MAYER: Dr. Ullberg, how do you avoid the loss of water-soluble labelled substances when stripping film is brought in contact with the section by the usual technique, i.e. under water?

Dr. ULLBERG: It is necessary to avoid water when attaching a section permanently to the emulsion. A way of combining a small thin tissue section with a photographic emulsion without isotope loss is to "dry mount" the section on an autoradiographic plate. A difficulty is then to get the sections to adhere to the photographic emulsion firmly enough to avoid dislocation during the development, fixing, washing and staining procedures. Another difficulty is to handle and to "stretch" a small easily curling section without water.

In our laboratory we catch the sections on "Scotch" cellulose tape when sectioning. Before the sections are mounted on the autoradiographic plate a very thin layer of egg-albumin-glycerin is applied to the emulsion and allowed to harden for a minute. After the autoradiographic exposure the plates are immersed in xylene which removes the tape but leaves the sections on the emulsion.