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COMMISSION ON RADIOCHEMISTRY AND NUCLEAR TECHNIQUES*

ISOTOPIC AND NUCLEAR ANALYTICAL TECHNIQUES IN BIOLOGICAL SYSTEMS A CRITICAL SURVEY

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Isotopic and nuclear analytical techniques in biological systems: A critical survey

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INTRODUCTION

The survey has been initiated by the interdivisional Commission on Radiochemistry and Nuclear Techniques (V.7) in cooperation with the Clinical Chemistry Division (VII). The Commission identified the techniques of interest, requested corresponding critical papers from experts in each field, reviewed the papers, and ultimately compiled them into a series of articles presented below. The first part of this survey includes articles on the following nuclear techniques which are currently available for qualitative and/or quantitative analysis of biological systems: autoradiography (macro-, micro-, and electron microscopic scale), postlabeling, radioimmunoassay and analogous binding assays, positron emission tomography (PET), particle-induced X-ray emission (PIXE), the perturbed angular correlation method (PAC), and Mössbauer spectrometry. Evaluation of the biological applications of isotope dilution, activation analysis, and other isotopic and nuclear analytical techniques will be published in the near future. The 'biological systems' considered include humans, animals, plants, and microbes, as well as cell-free *in vitro* models. The main aim of this survey is to provide biologists, clinical chemists, and environmental chemists, as well as analytical chemists, radiochemists, and others, with basic information, appraisal, and advice concerning the applicability, advantages, disadvantages, and development perspectives of the individual techniques. If necessary, this survey can be easily extended onto other newly developing isotopic and nuclear analytical techniques. Therefore, any comments and suggestions on this matter conveyed directly to the Editor will be greatly appreciated.

I. MACRO- AND MICRO-AUTORADIOGRAPHY

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Abstract - Autoradiography is an old technique which is continuously used and for which several new applications have been found in recent years, e.g., *in situ* hybridization, the use of short-lived radionuclides (^{11}C, ^{18}F), double-tracer autoradiography, and neutron capture radiography. The technique has been substantially improved by computerized image analysis and processing of the autoradiograms. Although some applications may be replaced by alternative methods, one can foresee that autoradiography also in the future will serve as a powerful tool in various areas of biological research.

Introduction

Autoradiography is performed by apposing a photographic emulsion to a material containing radioactivity. The detector is the silver halide grain of the photographic emulsion. There is a cumulative recording of charged particles in the vicinity of the radiation source. Compared to other biological radioisotope techniques, autoradiography can provide more detailed information on the localization or binding of radioactive materials in tissues.

Autoradiography can be used *in vivo* or *in vitro* for a number of applications (see Table 1). *In vivo*, a considerable range of substances, both

Table 1. List of references on original articles and/or reviews dealing with autoradiography techniques and applications.

Techniques	References
Apposition autoradiography	
(a) Whole-body autoradiography (cryostat sectioning on tape)	3, 4, 34
(b) Cryostat sectioning onto glass slides	7, 32, 34
(c) Low temperature autoradiography (apposition of blocks)	5, 6
Permanent contact autoradiography	
(a) Based on fixed and embedded tissues:	
(1) dipping and stripping autoradiography	2, 34
(b) Based on frozen tissues:	
(1) Thaw-mount autoradiography	2, 34
(2) Dry-mount autoradiography	7, 34
(3) Freeze-drying, paraffin or resin embedding, sectioning on tape	10, 34
(c) Hinged coverslip method	8, 9
Examples of applications	
Drug distribution	4
Incorporation of precursors in macromolecules (DNA, RNA, proteins, glycoproteins, lipids)	7, 35
In situ hybridization	7, 11-18
Glucose utilization	7, 36
Uptake of transmitters or precursors	7
In vitro receptor autoradiography	7, 9, 19
Blood flow studies	37
Localization of bioactivation products of xenobiotics	6
Studies on axoplasmic transport	7

physiological and foreign, are being used under varying conditions and with varying aims. For example, labeled thymidine can be used as a DNA precursor for study of DNA synthesis and cell proliferation, uridine can be used for study of RNA synthesis, and amino acids for study of protein metabolism. Other examples are labeled hormone precursors and hormones in order to study sites of hormone formation and to localize hormone receptors; glucose or 2-deoxyglucose for the study of glucose utilization in the nervous system and in extraneuronal tissues; transmitter substances or transmitter precursors in order to identify catecholaminergic, serotoninergic, cholinergic, GABA-ergic or other neurons. For foreign substances, it can be of interest to study the accumulation in target sites. For example, compounds which are accumulated and retained in fetal organs can give an indication of sites of teratological effects. Chemical carcinogens and other toxic compounds may undergo metabolic activation in the tissues, and autoradiographic techniques can be used to localize the sites of the bioactivation (ref. 1).

There are also important applications for autoradiography *in vitro*. One is visualization of various receptors for hormones and transmitter substances by incubations of the tissue sections with labeled ligands for the pertinent receptor sites. Others are the visualization of specific messenger RNAs by incubations of the tissues with labeled complementary nucleic acids (*in situ* hybridization) and radioimmuno-cytochemistry for the visualization of binding sites of antibodies, using labeled primary or secondary antibodies or labeled protein A.

There are some limitations of the autoradiography technique that are often brought up when it is critically evaluated. One is the fact that it is a label which is registered, not necessarily the original substance applied to the animal or specimen. This should always be kept in mind when autoradiograms are interpreted. The phenomenon that compounds are transformed in the body and thus sometimes lose the label, may however also be used to get specific information on biotransformation. If, e.g., a compound is specifically labeled, such as in a methyl group, and the removal of which is necessary for the biological activity of the compound, then this may be used in the study on rate of accumulation in a certain cell type and the rate of transformation of the compound in that cell.

The risk of misinterpretations in autoradiography due to chemography (positive or negative), fading of latent image or other artifacts, have been dealt with in detail previously (ref. 2). The old problem related to quantitation in autoradiography has been greatly improved with the development of computerized densitometry (for macroautoradiography) and counting of silver grains (microautoradiography). This however at the same time means that relatively expensive equipment is needed.

Autoradiography techniques are relatively laborious. In addition, they require long time for exposure. Critical evaluations of the various techniques are discussed currently in the text and in the Concluding Remarks below.

Autoradiographic techniques

Autoradiography can be performed as (a) apposition autoradiography, or (b) permanent contact autoradiography.

(a) Apposition autoradiography is used mainly for large specimens, such as sections through entire animals or whole brains. One application is the technique of whole-body autoradiography, which was introduced by Ullberg (ref. 3) and has since then been improved and extensively used (ref. 4). Animals are frozen into blocks of aqueous carboxymethyl cellulose (CMC) gel. In order to get intact sections, a tape is adhered to the surface of the block before a section is cut. The sections are freeze-dried and then pressed onto X-ray film for exposure. The film and the section are separated, the film is developed, and the section may be stained for identification of the tissues.

This procedure makes possible the study of diffusable substances which may otherwise be relocalized in the tissues during conventional histological processings. Even highly lipid-soluble compounds may be studied if the procedure is carried out at a temperature below ± 0°C. If volatile substances are studied, low-temperature autoradiography (-70°C) (refs. 5, 6), using thick sections taken by a rotary saw, can be performed to avoid

evaporation of the labeled material. To register firmly bound radioactivity, e.g., metabolites of xenobiotics bound to the tissues, the tape-fastened sections can be washed in various solvents before the autoradiographic procedure.

Cryostat sections can also be transferred onto glass slides (without the use of tape). This may be advantageous when studying receptor binding of radioligands *in vitro* (reviewed in refs. 2, 7).

Apposition techniques, combined with work with frozen sections, require cryostate equipment and/or freeze-room and combination freeze-room/dark-room facilities. Apposition autoradiography of large specimens upon registration of volatile substances further requires a precision circular saw in the freeze-room. Much of this work is inconvenient, and gloved hands are usually not very flexible.

(b) Permanent contact autoradiography is used mainly for cellular or subcellular light and electron microscopic localization of labeled materials (ref. 2). The biological specimen and the photographic emulsion are permanently combined. After exposure, the unit is passed through the photographic processing and through histological stain liquids. Preferably fixed and embedded tissues are used. The slides carrying the sections can be dipped in liquid nuclear emulsion. Alternatively, the "stripping film technique" can be used, in which film floated onto a surface of water is picked up to cover the section on a slide.

If diffusable substances are studied, sections of frozen tissues can be taken in a cryomicrotome and then be mounted onto a nuclear plate (glass slide dipped in nuclear emulsion) (ref. 7). An alternative to using nuclear plates is to mount the section on an ordinary glass slide and then apply the film to the section by a hinged coverglass already covered by an emulsion (refs. 8, 9). Some investigators prefer freeze-drying and impregnation of the tissue pieces with paraffin or resin (which however may involve risks for fat-soluble compounds) before the sectioning (ref. 10). The most tricky part for these methods is generally to get sections to stick well enough to the photographic plates, so that they do not change position or even get lost during the photographic processing.

Applications

As already mentioned in the Introduction, there has been a wide range of applications of the various autoradiography techniques. Table 1 lists a number of these applications and gives references to central and recent publications in the pertinent areas. Therefore, we will go into detail only with some more recent applications which may not have been covered so well in the literature.

In situ hybridization. Hybridization techniques have been applied to histological sections since the early 1980's. Gee and coworkers (ref. 11) identified mRNA in specific hormone-producing neurons by use of a complementary DNA (cDNA) probe in parallel with immunocytochemistry. Later, cRNA and oligodeoxyribonucleotide probes (around 50 bases long) which can be synthesized automatically have been used. The probes are labeled with [^{35}S]thiodeoxyadenosine triphosphate or other radionucleotides (^{3}H, ^{32}P, ^{125}I) (refs. 12, 13). *In situ* hybridization can also be subjected to quantitative analysis (ref. 14). The technique has been widely utilized to identify polypeptide hormone-producing cells, e.g., in the CNS (ref. 7) and in the immune system. Furthermore, intra- and extracellular proteins, viral products in cells, etc., can be visualized. Double-isotope technique has been exploited, utilizing ^{35}S- and ^{3}H-labeled probes on the same section, and then two layers of emulsion separated by a thin mylar film were used to discriminate ^{3}H radiation (ref. 15). The last few years, *in situ* hybridization has been extensively used to determine the spatial and temporal expression of genes during embryonic development important for pattern formation and segmentation processes, especially in Drosophila but also in mammals (mainly mice). One example is the homeobox gene family, the products of which act as sequence-specific DNA-binding proteins, regulating the expression of structural genes (refs. 16, 17).

The results obtained with *in situ* hybridization techniques have to be interpreted with caution and should ideally be accompanied by the identification of the pertinent protein or polypeptide by other means (e.g., immunohistochemistry), since mRNA may have a short biological half-life or

otherwise not being properly translated. Also proper controls are essential, since there are many possible sources of non-specific binding (ref. 12). Lately, non-radioactive methods have been developed to label DNA- and mRNA-binding probes, such as the avidin-biotin complex (ref. 18).

In situ hybridization autoradiography has already given extensive information on gene expression in specific cells, and will continue to do so. Due to the time factor as mentioned above, and the development of labeling of probes by which they can be directly visualized, one can foresee a development where autoradiography may be gradually replaced by these other techniques.

Short-lived radionuclides. The use of short-lived radionuclides, e.g., ^{11}C($T_{½}$ = 20 min) and ^{18}F ($T_{½}$ = 110 min), in autoradiography has recently become of practical interest as a complementary technique to positron emission tomography (PET). Results from autoradiography studies may facilitate the interpretation of PET images, since the resolution in autoradiography is about 50 times better (ref. 19). As the PET technique is now expanding, the availability of ^{11}C- and ^{18}F-labeled substances will increase, making possible a substantial decrease of exposure times, and also of double-isotope studies (see below).

As a principle, autoradiography with short-lived radionuclides is similar to conventional autoradiography, but the various steps of the technique have to be speeded up (ref. 20). The radiodoses needed are higher (about 50 µCi/g body weight) and the extent of the observation period of the experimental animals after administration of the labeled compounds limited (less than 3 half-lives of the radioisotope). The short exposure time is of course an advantage with respect to planning of, e.g., current PET investigations.

It is also possible to obtain very high specific activity with, e.g., ^{11}C -- usually the order 200-2000 Ci/mmol (ref. 21). This is of special importance in work with extremely toxic compounds, as well as with substances having strong physiological effects, e.g., peptide hormones.

Substances labeled with short-lived radionuclides may also be used in double-tracer autoradiographic studies (cf. below). The difference in decay between a long-lived radionuclide and a short-lived one is then used for discrimination (refs. 20, 22-24).

Double-tracer autoradiography. Double-tracer techniques permit mapping of the distribution of two radiolabeled substances simultaneously in the same experimental animal/specimen. The main advantage is that systemic errors due to biological variations may be avoided, for instance when studying two different compounds in animals transplanted with tumors.

The most promising approach in this regard has turned out to be the use of one short- and one long-lived radioisotope, e.g., ^{11}C and ^{14}C (refs. 20, 24) (cf. above). Two separate exposures are performed. The first, being very short (up to three half-lives of the short-lived isotope, i.e., one hour for ^{11}C) and providing the distribution image of solely the short-lived nuclide; the contribution from the other nuclide is negligible. The next exposure, which takes place when the short-lived isotope is practically disintegrated, is rather prolonged and gives autoradiograms of the long-lived isotope.

Double-tracer techniques, mainly based on differences in radiation ranges of the two radionuclides, e.g., ^{3}H and ^{14}C, are also used (refs. 2, 15, 25). Thin mylar sheets, interspaced between the section and the film during the exposure, are used to absorb the radiation of the low-energetic tracer (^{3}H) but not of the other nuclide, giving the distribution image of the latter (i.e., ^{14}C). The re-exposure, without the mylar sheet, will then give the combined image of the two nuclides. The detailed interpretation of the autoradiograms is, in practice, difficult. It is facilitated by computerized image analysis (cf. below) by the subtraction of corresponding images of the two exposures from each other. Due to the use of the mylar sheet as well as the fact that image analysis has to be used, the resolution in this technique is reduced.

Double-tracer autoradiography using differences in solubility of two (differently labeled) substances has also been tried (ref. 26), but this approach is only restricted to experiments where one of the substances may easily be removed from the section by extraction after the first exposure.

Quantitative neutron capture radiography. An interesting alternative to radionuclides in autoradiography is stable isotopes, e.g., ^{10}B, which can be transformed directly in the freeze-dried sections to radioemitters by neutron capture and be registered (refs. 27-29). ^{10}B undergoes instantaneous fission when exposed to thermal neutrons according to the reaction ^{10}B (n, α) ^{7}Li. The emitted α particles may be registered by special plastic film detectors, made of cellulose nitrate (Kodak-Pathé LR 115, types I or II), which are closely apposed to the sections during the neutron exposure. The cellulose nitrate films are "developed" by etching in hot aqueous NaOH. The microtracks, which are formed, can be recorded under microscope. Absolute quantification of the boron concentration in various tissues may be obtained by the simultaneous exposure of the section and standards, containing known amounts of boron (ref. 29). Normally, the total track areas are measured and compared. The etching of the detectors has turned out to be the most critical step in the technique. If the track density is high, there is a tendency that increased etching causes fusion and overlap of separate holes (tracks), with an underestimation of the total track area. The problem may be controlled and restrained by test etchings for optimizing the final etching time, since the same film can be etched several times. Natural boron consists of 19.6% ^{10}B, but 95% isotopically enriched ^{10}B precursors for the boron labeling of experimental substances, are commercially available (Eagel Picher Research Lab., Miami, USA). ^{10}B-Labeled substances are clinically interesting for boron neutron capture therapy of tumors (ref. 28).

A prerequisite for the neutron capture radiography technique is that a neutron source (reactor) is available. A network of collaboration in this field has been established.

Computerized image analysis. The quantitative determination of the concentration of radioactivity in various tissues of whole-body sections has been substantially improved during the last decade by computer-assisted analysis of the optical densities of the autoradiograms (ref. 30). Image processing systems in this regard are either based on microdensitometric readings or video scanning techniques, which are cheaper, faster, and have the advantage of an easily altered magnifying power. Ramm and co-workers (ref. 31) have shown that a corrected video camera normally provides measurements of sufficient accuracy for use in quantitative autoradiography.

So far, most of the applications and image processing routines have been focused on brain analyses (ref. 32). Video-based computerized image processing systems have been developed in various laboratories during the last decade. They consist of commercially available components, normally including a video camera and a video interface, a microcomputer and graphic equipment, monitors, picture memory, digitizing pad, etc. (cf. ref. 30). The programs, which are designed for the analysis of whole-body autoradiograms, allow management of contrast, pseudocoloring, etc. A central program of our system (ref. 30) that has turned out to be particularly useful, displays the optical density histogram for an area of interest of the autoradiogram, and from this histogram a certain range of densities may be selected. The corresponding picture elements (pixels) are then indicated (e.g., in red) on the image display, and their average density is calculated. The procedure permits interactive marking and analysis of any structure of the autoradiogram, even very tiny and irregular distribution patterns. By other programs, it is possible to rotate and translate pictures, stored in the memory, and to superimpose two images for comparison (including pictures of the autoradiogram and the corresponding section). The superimposed pictures may be subtracted from each other, or added or divided, pixel by pixel. Various programs for image enhancement have also been developed.

The previous image analysis systems were quite expensive, especially the computer hardware, and many commercially available programs were usually designed for general image processing applications, making them difficult to handle for the analyses of autoradiograms. During the last few years, however, relatively inexpensive systems have been developed and marketed.

Concluding remarks

Autoradiography, which offers the detection of radioactivity in tissues, is a technique with relatively high sensitivity. The sensitivity, however, varies greatly depending on the radionuclide used and the type of investigation: e.g., the density of receptors if radioactive receptor

ligands are used; the amount of RNA if *in situ* hybridization is performed; the number of adducts if covalent binding of bioactivation products are studied. These are also good examples of applications where exclusive information on the level of radioactivity in specific cells can be created by autoradiography as compared to techniques where an average value within a given amount of tissue is presented.

In many instances, so minute structures are studied that it is practically impossible to dissect them for other types of analyses. This may be true, e.g., in studies on the localization of drugs during embryogenesis (ref. 33). With respect to whole-body (macro) autoradiography, so much information is obtained that no other technique could possibly be competitive.

Among the difficulties and disadvantages can be mentioned those related to quantitation, such as artifacts in the form of positive and negative chemography, due to chemical factors in the specimen or image fading, especially during long exposure periods or improper storage conditions. Others are physical blackening of the film, e.g., at the edges of specimens or upon improper handling. The problems of quantitation have many other aspects. Some are related to technical problems, such as the use of proper radioactive standards or the use of complicated statistical methods. Others have to do with geometrical limitations, e.g., the underrepresentation of minute structures due to a relatively long range of the radioactive particles. The latter also contributes to a low resolution, which may be better with, e.g., immunohistochemistry.

Some of the disadvantages with autoradiography only concern problems of specific applications. *In vivo* studies on drug distribution may give misleading results, if the radioactive label is lost from the principal molecule and is excreted or participates in the intermediary metabolism (e.g., one carbon fragment if ^{14}C is used). Chemical analysis of extracts from tissues is therefore often advisable.

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II. ELECTRON MICROSCOPE AUTORADIOGRAPHY

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Abstract - Electron microscope autoradiography provides information on the ultrastructural localization of radioisotopes in biological tissue, and involves *in vivo* labelling, processing for microscopy and qualitative/quantitative analysis of the final preparation. Resolution and sensitivity of the technique depend on the energy of radioisotope emission and various aspects of the autoradiographic preparation.

Introduction

Electron microscope autoradiography provides a technique of considerable potential for the high resolution analysis of radioisotopes within biological samples, and involves the detection of these molecules in electron microscope preparations by recording their sensitization of an applied layer of photographic emulsion.

The major object of this review is to consider the fundamental potential of the technique in relation to different aspects of preparation and analysis. The use of autoradiography with isotopes of different emission energies will be considered, and some applications of the technique will be described in reference to one particular isotope - ^{63}Ni.

In general, autoradiographic procedures can be divided into three main aspects:

(1) Labelling of cells or tissues with radioactive materials

The radioactive isotope may occur either as an isolated molecule, e.g., ^{63}Ni and ^{45}Ca cations, or as part of a more complex molecule, where the nuclide (e.g., ^{3}H, ^{125}I) is in a bound state. In the latter case, the radioactive molecule may act as a specific precursor for the synthesis of a particular macromolecule (e.g., [^{3}H]thymidine as a precursor for DNA

synthesis) or it may act as a specific probe for particular receptor molecules (e.g., use of ^{125}I proteins and peptides in receptor and toxin studies, see ref. 1).

In some cases the labelling procedure may also involve the sequential use of non-radioactive precursors (pulse-chase experiment), particularly where the location of intracellular synthesis and route of transport are being studied.

(2) Specimen preparation

This conventionally involves fixation, embedding and sectioning, followed by coating with photographic emulsion and incubation in the dark for a specified length of time. The preparation is finally photographically developed and fixed prior to microscopic observation.

(3) Examination and interpretation

The location and abundance of silver grains lying over the specimen can be assessed qualitatively by direct observation and quantitatively by statistical analysis.

High resolution autoradiography can be separated into two major types, depending on whether it is in the scanning electron microscope (SEM) or transmission electron microscope (TEM) mode. The major part of this article will be devoted to TEM autoradiography since most studies have used this approach.

A. Scanning electron microscope autoradiography

This is a relatively new field, and holds considerable promise for the future, with the increasing use of SEM in the biological sciences. The first reported use of this technique was by Paul, Grobe and Zimmer (ref. 2) working on the localised occurrence of ^{3}H in cellulose fibre, and the first published biological application was by Hodges and Muir (ref. 3), looking at the localisation of [^{3}H]thymidine in bladder explant sections.

Technique

1) *Labelling procedures.* SEM autoradiography has been carried out mainly on monolayers of single cell cultures, including algae (ref. 4) and cultured animal cells (refs. 5, 6). The studies have largely involved the use of tritiated compounds, though Junger and Bachmann (ref. 6) used ^{125}I-labelled insulin.

2) *Specimen preparation.* One of the major problems with SEM autoradiography is the potential induction of specimen damage by wet emulsion, air-drying during the incubation period (photographic exposure), then wet photographic processing prior to final dehydration. Most workers have used fixed material, which has the advantage of stabilizing cell structure throughout the preparation schedule.

The sequence of post-labelling procedures is typically complex and may involve (i) Fixation, (ii) Dehydration in ethanol followed by critical point drying, (iii) Application of photographic emulsion, (iv) Incubation period (up to 2-3 months), (v) Photographic processing, (vi) Removal of gelatin, then (vii) Final dehydration and critical point drying. The application of emulsion may be either by stripping film (ref. 6) or by dipping in liquid emulsion (ref. 3). Removal of gelatin after photographic processing is essential, to reveal details of surface structure, but must be carried out in such a way as to avoid displacement of the silver grains. Procedures for doing this include the application of trypsin, warm water (ref. 4), and NaOH (ref. 3).

3) *Examination and interpretation.* The major distinction between scanning and transmission electron microscope autoradiography lies in the bulk nature of the specimen in the former case. In SEM autoradiography, the silver grains produced in the surface emulsion may thus be derived from internal regions of the specimen or from cell surface radioactivity. Uptake of [^{3}H]thymidine, for example, will lead to labelling of DNA-synthetic nuclei within cells and result in surface accumulations of silver grains in relation to these internal sites (refs. 3, 5). The use of cell surface labels, such as [^{3}H]glucosamine (ref. 5) and [^{125}I]insulin (ref. 6) can lead to specific information on receptor occurrence and localization in the plasmalemma/glycocalyx complex.

B. Transmission electron microscope autoradiography

In contrast to SEM autoradiography, TEM preparations typically involve the use of ultrathin specimens. These have the advantage over bulk specimens of high spatial resolution, but the small amount of label present may require long exposure periods for adequate yield of silver grains. Although the great majority of studies have used ultrathin sections, other types of material have also been employed, including whole-amount (particulate) samples (ref. 7) and freeze-fracture preparations (refs. 8, 9).

Technique

1) *Labelling procedures*. TEM autoradiography has been used with a wide range of labelled compounds and a wide range of isotopes, including ^{3}H, ^{14}C, ^{63}Ni, ^{45}Ca, ^{125}I, and ^{32}P (see later). The relatively low yield of silver grains obtained with TEM autoradiographic preparations means that specimens should be as heavily labelled as possible. For this reason it is always useful to carry out a preliminary autoradiographic check at the light microscope level to ensure that material is adequately labelled before proceeding on the long-term TEM study.

2) *Specimen preparation*. This involves three main aspects: preparation of the ultrathin specimen, coating with emulsion, and photographic processing.

a) Ultrathin specimen. In conventional TEM autoradiography, labelled cells are fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in an ethanol series then embedded in resin and sectioned. The use of such chemical techniques has the advantages of ease of preparation and retention of full ultrastructural detail in the final preparation, but the disadvantage that soluble constituents are lost from the cells. This approach can thus only be used where bound or insoluble labelling is being investigated. Care should be taken in the use of fixatives where free amino acid uptake is being monitored, since artificial binding of amino acids to proteins may occur as an artefact (ref. 10).

If soluble label is to be retained and monitored in the autoradiography, cryotechniques have to be employed. The most direct approach is to use freeze-dried cryosections (ref. 11).

b) Coating with emulsion. To achieve optimal resolution, where silver grains show maximal localisation to sites of radioactivity, the emulsion must be applied as a monolayer (i.e., a single layer of silver halide crystals) to the section (refs. 12, 13). In general, a monolayer of photographic emulsion can be applied either as a stripping film or as a liquid emulsion to sections mounted on a flat substrate (e.g., celloidin-coated glass slide, ref. 14) or on an EM grid. In the latter case, a liquid emulsion is mixed then collected on a wire loop, which is drained and passed over EM grids bearing sections.

The choice of emulsion is also important for optimal resolution, since the smaller the mean size of the silver halide crystals the greater the resolution. This arises because the site of silver grain initiation (the latent image) typically occurs at a different place in the silver halide crystal from the site of initial sensitisation. Clearly, the greater the size of the silver halide crystal, the greater the possible distance between the two sites and the greater the scatter of silver grains. A useful amount of the theory of the photographic process is provided by Carroll *et al.* (ref. 15). A number of small grain emulsions are available for TEM autoradiography, ranging in grain size from 0.14 to 0.26 μm in diameter (ref. 16).

As with light microscope autoradiography, there is a danger in TEM preparations that chemicals in the specimen may diffuse into the photographic emulsion and either promote the non-specific formation of silver grains (resulting in increased background) or prevent the formation of label-sensitised grains (resulting in loss of image). Such chemographic effects vary with different emulsions (ref. 16), and may be prevented by placing a film of carbon (ref. 17) or other material (ref. 18) over the specimen prior to coating with emulsion.

As noted earlier, a problem that may frequently arise with TEM autoradiographs is the relative low yield of silver grains over the specimen. This arises mainly due to the ultrathin nature of the specimen combined with the requirement of a monolayer of small diameter silver halide

crystals. One novel approach to increasing silver grain yield with a particular specimen is to place a thin scintillator film between the specimen and support film or between the specimen and photographic emulsion (refs. 7, 19). This approach increases the photographic efficiency of silver grain formation, but has potentially adverse effects on the resolution of the technique.

The exposure time of TEM autoradiographs is substantially longer than light microscope preparations, and is normally in the order of 2-3 months. Longer exposures than this are not generally beneficial due to latent image fading (ref. 20), and also because the build-up in background count outweighs the theoretical increase in specimen-sensitised grains.

c) Photographic processing. This follows the standard procedure of development, stop bath (optional) and photographic fixation. Removal of excess emulsion (as in SEM autoradiography) is not normally required. The conditions of photographic development are the most crucial aspect of the process in determining the final quality of the preparation, affecting both the number of silver grains produced and the size and appearance of the grains. Conventional developers such as Kodak D19 produce a high silver grain yield, but also large grains, which appear as complex "wires" under the electron microscope. Use of fine grain chemical developers such as Microdol-X produces small spherical silver grains, resulting in enhanced resolution. The need to keep silver grain size as small as possible for maximal resolution has led to the use of more refined development techniques, including the use of physical developers (ref. 21) and gold latensification (ref. 22). The latter technique involves deposition of a fine layer of gold around the latent image, followed by chemical development, and results in extremely small silver grains.

3) *Examination and interpretation.* Where TEM autoradiography has been successfully carried out, individual silver grains can be clearly seen lying over ultrathin sections in which fine-structural features are well-defined. Direct examination of the autoradiographs can give useful qualitative information on the occurrence and localisation of silver grains, but a more thorough approach requires the application of quantitative methods, with an understanding of the quantitative resolution of the technique.

a) Quantitative analysis of autoradiographs. The major objective of quantitative analysis of TEM autoradiographs is to determine the mean level of radioactivity in different parts of the cell. Although silver grains can be readily observed in relation to cell ultrastructure, the quantitative assessment of TEM autoradiographs is not straightforward for two reasons. Firstly, silver grains have a finite size and may lie across different parts of the cell, making allocation difficult. Secondly, the presence of a silver grain over a particular cell compartment does not imply that the grain is necessarily derived by sensitisation from that part of the cell. It may have arisen as part of the nonspecific 'background' count or by lateral sensitisation from another part of the cell. Because of the uncertainty in the significance of occurrence of individual grains, the quantitative analysis of TEM autoradiographs becomes a statistical problem. This analysis must clearly involve an estimation of background contribution to grain counts, and must also take into account the degree of lateral sensitisation (resolution) from radioactive sources in the specimen. A wide range of studies have now been published on image analysis in TEM autoradiographs (see e.g., refs. 23, 24), and with increasing sophistication, have involved the use of computer technology. An example of this is provided by the quantitation approach of Downs and Williams (ref. 25), where a computer based iterative scheme is used to relate the distribution of silver grains over the autoradiograph to the most appropriate "model."

b) Resolution. Photographic resolution in TEM autoradiography relates to the spread of silver grains around the sites of radioactivity in the specimen, and has been experimentally defined in a number of ways. Caro (ref. 12) defined resolution as the radius of a circle around a point source of radiation which enclosed 50% of the silver grains derived from the source. Using T-2 phage and *Bacillus subtilis* labelled with [^{3}H]thymidine, the point resolution was determined at 0.1μm. Salpeter considered resolution in relation to a solid line source of [^{3}H]polystyrene (with Ilford L4 emulsion and 50nm-thick sections), and found that 50% of the silver grains occurred within a distance of 0.13μm from the centre of the source. This distance was defined as the half distance of resolution or HD. Using this data, Salpeter *et al.* (ref. 26) and Salpeter & Salpeter (ref. 27) have

generated theoretical 'universal curves' for grain density distribution around sources of various geometrical shapes. Salpeter's HD (line resolution) values for ^{3}H have been widely used for analysing results from various biological samples.

High energy isotopes

Although the great majority of TEM autoradiographic studies involve the use of ^{3}H, other (higher energy) isotopes are increasingly being used (Table I).

Table I. Maximum beta emission energy of isotopes used for EM autoradiography

Isotope:	^{3}H	^{125}I	^{63}Ni	^{14}C	^{35}S	^{45}Ca	^{32}P
Emission energy (keV):	18	(*)	67	155	167	254	1072

(*) ^{125}I emits Auger electrons in the ranges 0.5-3.5 keV and 22.7-34.2 keV (ref. 1).

The energy of the beta-radiation emitted by a particular isotope is important in determining both the resolution and the sensitivity of the autoradiographic preparation. With increase in energy, there is a decrease in resolution (due to increased emulsion penetration) and a decrease in sensitivity (due to decreased energy loss at the beginning of particle trajectories). This is shown by comparison of ^{3}H and ^{32}P, at extreme ends of the isotope range, where there is a drop in point resolution from 0.1 to 0.3 μm, and a decrease in the number of halide crystals sensitised from 20 grains/100 emissions to 2.5/100 emissions (ref. 28).

With some isotopes, the resolution is considerably better than might be expected form the mean emission level, since it is determined more by low energy bands in the emission spectrum. In the case of ^{125}I, for example, the line resolution (expressed as HD units) is better than that for ^{3}H since it is determined largely by Auger electrons in the 0.5-3.5 keV range rather than by emissions of higher energy (ref. 1).

TEM autoradiographic studies have been carried out using ^{125}I (refs. 1, 29), ^{63}Ni (see below), ^{14}C (ref. 27), ^{45}Ca (ref. 30), and ^{32}P (ref. 28).

^{63}Ni autoradiography

Although ^{63}Ni has been used in a number of light microscope autoradiographic studies, relatively little work has been undertaken at the level of the electron microscope. Investigations have been carried out, however, on the incorporation of ^{63}Ni into dinoflagellate cells and bacteria, in both cases demonstrating a high degree of localisation of bound label to the chromatin.

1) *Dinoflagellate cells*. Electron microscope studies on the uptake of ^{63}Ni into the binucleate dinoflagellate *Glenodinium foliaceum* (refs. 31, 32) demonstrated clear localisation of bound label to the dinocaryotic (but not supernumerary) nucleus, and suggested further localisation within this to the dinoflagellate chromosomes. These observations corroborated earlier X-ray microanalytical demonstrations of the occurrence of transition metals (including nickel) in dinoflagellate chromatin generally (refs. 33, 34) and are of considerable interest in considering the organisation and phyllogeny (refs. 35, 36) of the dinoflagellate chromosomes.

2) *Bacterial cells*. Recent TEM autoradiographic studies (refs. 37-39) have demonstrated an active uptake of ^{63}Ni into laboratory-cultured cells of *Pseudomonas syringae* pv *tabaci*. Parallel scintillation studies showed that over 80% of the label present in the living cells was lost during fixation and ethanol-dehydration, with the remainder being retained in autoradiographs in bound form.

Scintillation counts on the level of ^{63}Ni in suspensions of bacteria fixed in different ways also provides useful information on the minimum levels of autoradiographic detectability (ref. 37). Retained levels of radioactivity were higher in suspensions of acetic alcohol-fixed compared

to glutaraldehyde-fixed cells, in agreement with the TEM autoradiographic results, where grains counts were substantial in acetic alcohol preparations but were approaching the limits of significance in glutaraldehyde fixed material. The mean level of incorporated label in the glutaraldehyde-fixed cells was about 0.2 μCi/10^{10} cells. With a specific activity of 11.6 μCi/mg, this is equivalent to a mass level of incorporated label of approximately 3 X 10^{11} nmol/cell or 7.4 X 10^4 Ni atoms per cell. Under these experimental conditions, this value is approaching the mean level of nickel detectability using TEM autoradiography.

With D19 developer, silver grains over bacteria were relatively large and showed no localisation to internal features. The use of gold latensification followed by physical development resulted in a much enhanced resolution, with small multiple silver grains showing clear localisation to central chromatin. This occurred either as a dense precipitate (acetic-alcohol fixation) or as coarse strands within the nucleoid (glutaraldehyde fixation).

Parallel studies using X-ray microanalysis showed that nickel could be routinely detected (with other cations) in extracted bacterial DNA (ref. 40), and in fixed (but not unfixed) whole cells grown in normal growth medium (ref. 41).

Future trends in electron microscope autoradiography

It seems likely that future trends in electron microscope autoradiography lie in more diverse and novel applications of the technique, with more sophisticated computer analysis of autoradiographs, rather than with any major advances in resolution or sensitivity. The use of autoradiography in conjunction with scanning electron microscopy, and the application of the technique to study the incorporation of high energy isotopes are areas of particular interest.

The ability to label cells in the living state and follow the time course of chemical transitions within the protoplasm offer a major advantage over other cytochemical techniques such as immunohistochemistry and X-ray microanalysis, which present rather a static view of the cell. The success of autoradiography depends to a large extent on the degree and specificity of labelling, and the future use of this technique will parallel the development of site-specific probes (labelled molecules) of high specific radioactivity. A good example of this is the use of newly-developed radiolabeled drugs and neurotransmitters to study the spatial and dynamic binding of these ligands to receptors in the nervous system (ref. 42).

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III. ^{32}P-POSTLABELING ANALYSIS OF MUTAGEN/CARCINOGEN-DNA ADDUCTS

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Abstract - Covalent binding of chemicals or their metabolites to DNA is a critical event in the initiation of chemical carcinogenesis. Measurement of covalent DNA adducts in model and human systems has become an important tool in mutation and cancer research. The recently developed, highly sensitive ^{32}P-postlabeling assay of DNA adducts and its applications are critically reviewed.

Basic features of assay

^{32}P-postlabeling analysis is a recently developed, highly sensitive method for the detection and measurement of covalent DNA adducts. Since the assay is based on the enzymatic incorporation of ^{32}P into DNA nucleotides, it does not require the carcinogens to be radiolabeled. Therefore, the method is suitable for DNA of humans exposed to environmental or occupational genotoxicants. The basic method (ref. 1) entails digestion of DNA with a mixture of micrococcal nuclease and spleen phosphodiesterase to 3'-mononucleotides, conversion of the digestion products to 5'-^{32}P-labeled 3',5'-bisphosphate derivatives with [γ-^{32}P]ATP as the donor of label and T4 polynucleotide kinase as the catalyst, separation of the labeled nucleotides by polyethyleneimine (PEI)-cellulose TLC, autoradiography to detect adducts and normal nucleotides, and quantitation by scintillation counting. A key feature of the assay is the class separation of aromatic or bulky/hydrophobic adducts from the usually large excess of normal DNA nucleotides; this is readily accomplished by PEI-cellulose TLC (refs. 2-6).

Various versions of the assay have been developed. The adducts may be labeled in the presence of the normal DNA nucleotides, or, alternatively, to increase the sensitivity of the assay, normal nucleotides are removed before ^{32}P-labeling of the adducts. For adduct enrichment, physical techniques such as butanol extraction (ref. 7) and HPLC (refs. 8, 9) or enzymatic techniques such as nuclease P1 post-digestion (ref. 10) have been described. In the nucleoside monophosphate postlabeling assay (ref. 11), adducts are purified as part of the initial DNA hydrolysis step and obtained in the form of dinucleotides which are subsequently converted to ^{32}P-labeled

5'-monophosphates. The enrichment procedures afford the greatest sensitivity of adduct detection (1 adduct in 10^9 - 10^{10} DNA nucleotides) and usually require 5-10 μg DNA per individual assay. Recoveries of certain adducts by the enrichment procedures depend on adduct structure (refs. 10-12).

Choice of procedure

The choice of labeling procedure depends on the amounts and types of adducts present in the DNA under study. If the adducts are labeled in the presence of an excess of [γ-^{32}P]ATP and normal nucleotides are not removed, the limit of detection of aromatic or hydrophobic adducts is 1 adduct in 3 x 10^7 nucleotides. This procedure, which affords quantitative labeling of both normal and adducted nucleotides, may be used to calibrate the other procedures. Labeling under ATP-deficient conditions enables the detection of many adducts without enrichment at levels as low as 1 in 10^9, as a consequence of preferential labeling of adducts over normal nucleotides (ref. 13).

The choice of chromatographic procedure is key to optimal adduct resolution. Ion-exchange TLC on PEI-cellulose has been used most extensively for this purpose. Concentrated electrolyte solutions containing 7-8.5 M urea (refs. 2-6) appear best suited as solvents for non-polar adducts containing polycyclic aromatic moieties, while more polar adducts are resolved with solvents containing lower concentrations of electrolytes and urea (refs. 5, 14, 15). In two-dimensional chromatography, optimal resolution is usually achieved with acidic solvents (pH 3.3-4) in the first dimension, followed by near neutral (pH 7-8) solvents in the second dimension. Derivatives of widely divergent polarities in a DNA sample may require several mapping systems.

Adduct quantitation

Adduct levels are calculated as relative adduct labeling (RAL) values, which represent the ratio of count rates of adducted nucleotides to count rates of total (adducted plus normal) nucleotides (refs. 2, 5, 6, 13). The radioactivity of normal nucleotides is evaluated by analyzing an aliquot of the labeled DNA digest. In procedures employing nuclease P1, where normal nucleotides are removed prior to labeling, the specific activity of [γ-^{32}P]ATP is utilized for RAL calculations (refs. 5, 10, 11). The RAL x 10^n value is equal to the number of adducts in 10^n DNA nucleotides. Microgram amounts of DNA are analyzed; thus the assay is well suited for the analysis of DNA lesions whenever limited amounts of cells or tissues are available. For example, transplacental DNA damage induced by carcinogen exposure of pregnant laboratory mice has been analyzed (ref. 16).

Applications

As reviewed by Gupta and Randerath (ref. 5), the assay has been successfully applied to a variety of mutagenic (genotoxic) as well as non-mutagenic carcinogens. ^{32}P-adduct measurements have been directed at DNA preparations modified by over 100 test chemicals, comprising arylamines, arylamides, nitroaromatics, nitrosamines, azo-compounds, dyestuffs, polynuclear aromatic hydrocarbons, heterocyclic polynuclear aromatics, epoxides and methylating agents, as well as mycotoxins, alkenylbenzenes, and antibiotics. In addition, DNA alterations associated with exposures to complex mixtures and unidentified sources have been detected by ^{32}P-postlabeling. Among the non-mutagenic carcinogens are estrogens (refs. 17, 18), 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) (ref. 19), and choline-deficient diet (ref. 20). In addition, age-dependent DNA modifications (I-compounds) are detected by ^{32}P-postlabeling in animals that have not been knowingly exposed to mutagens/carcinogens (refs. 15, 21, 22). In humans, the ^{32}P-postlabeling assay has been applied to DNA specimens from cigarette smokers (refs. 23-25) and iron foundry workers (ref. 26). Estimation of total aromatic adduct levels in exposed individuals gave values of 1 adduct in 10^6-10^8 DNA nucleotides. These values are similar to the total levels of persistent adducts in tissues of animals after exposure to initiating or carcinogenic doses of authentic aromatic genotoxicants.

Current applications and prospects

The ^{32}P-postlabeling assay is being used mostly to analyze DNA lesions arising from exposure to bulky/hydrophobic carcinogens (e.g., benzo[*a*]pyrene, 4-aminobiphenyl, safrole, protein pyrolysis products,

cigarette smoke constituents) because the resulting adducts are readily purified by chromatography from normal DNA nucleotides. Special enrichment techniques are required for small alkylated adducts (refs. 6, 9). Main advantages of the ^{32}P-postlabeling assay are its rather general applicability to a diverse spectrum of DNA modifications and its great sensitivity which exceeds the sensitivity of other known methods for DNA adduct analysis by several orders of magnitude. On the other hand, the assay is not suitable for structural identification of the adducts unless model compounds are available. Current physical methods such as mass spectrometry are frequently not sensitive enough to identify the small amounts of DNA adducts or I-compounds isolated from biological sources.

Important future developments will include improvements in ^{32}P-postlabeling of small adducts, refinement of sensitive methods for structural characterization of trace amounts of adducts, and techniques for determining the exact location of lesions in subfractions of genomic DNA or in specific genes. Perhaps the most important applications of the assay will address questions of mechanisms of carcinogenesis and of dosimetry (ref. 27) and risk assessment (e.g., extrapolation to humans of animal data concerning relations between DNA adducts and cancer incidence or other adverse health effects).

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IV. RADIOIMMUNOASSAY, IMMUNORADIOMETRIC ASSAY AND ANALOGOUS BINDING ASSAYS

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Abstract - The terminology, basic physicochemical and immunologic principles, and performance characteristics of radioisotopically-based immunoassays are presented and evaluated. Development trends and perspectives of radioimmunoassays are critically discussed.

Nomenclature; area of application

The term "radioimmunoassay" (RIA) is occasionally employed in a general sense to describe *any* immunoassay procedure in which the immunological reaction between the two primary reagents involved, i.e., analyte and antibody, is revealed using either "tracer" analyte or antibody labelled with a radioisotope. However, its use is usually (and preferably) restricted to assays in which the trace analyte is so labelled, the term "immunoreactive assay" (IRMA) being applied to methods relying on the use of radiolabelled antibody. Both forms of immunoassay fall within the class of techniques - frequently described as "binding assays" - which rely on observation of the reaction between the analyte and a specific binding substance, the latter being generally of biologic origin and typically a specific binding protein. For example, hormone receptors have been used in this fashion, in techniques generally referred to as "radioreceptor assays". Nevertheless, antibodies have, in practice, comprised the group of binding proteins most widely exploited in this context, primarily because they can be generated conveniently and in large amount (either by conventional *in vivo* methods or, more recently, by the *in vitro* techniques pioneered by Milstein and Köhler (ref. 1) against a broad range of substances of biological interest, including high molecular weight substances, e.g., proteins polypeptides, viral particles, oligonucleotides, as well as substances of relatively small molecular size ("haptens") such as drugs, steroid and thyroid hormones, vitamins, etc. However, naturally-occurring specific binding proteins continue to be used in "protein binding" assays relying on identical analytical principles, e.g., in assays for vitamin D, vitamin B_{12}, folic acid and (occasionally) thyroxine, albeit the availability of specific antibodies against most of these substances has resulted in the progressive abandonment of this particular approach. More recently, binding assays essentially identical in principle to RIA and IRMA, but relying on DNA "probes" as the specific binding reagent, have been introduced, though they are restricted in application to the detection and/or measurement of complementary nucleotide sequences, and have not as yet achieved the widespread usage or commercial importance of antibody-based methodologies.

Immunoassays characteristically display very high "structural specificity", reflecting the extreme ability of appropriately selected antibodies to distinguish and bind to antigenic sites (epitopes) on particular analyte molecules. The use of a radioisotope such as ^{125}I or ^{3}H (or, in the case of DNA probes, ^{32}P) to label either the antibody or tracer analyte permits binding reactions between very small numbers of molecules to be readily monitored, providing the basis of these methods' high sensitivity. The combination of their high specificity and sensitivity, coupled with their

relative simplicity, underlies the ubiquitous use of radioisotopically-based immunoassays in medicine and biology (both in research and diagnosis)and increasingly in many other fields (e.g., the food industry, agriculture, forensic science environmental monitoring, etc.), and methods of this genre have now been described for thousands of substances of biological importance.

Basic design principles underlying radioisotopically-based immunoassays

Radioisotopically-based immunoassays conform to a variety of differing designs, some of which profoundly affect their performance characteristics. All immunoassays essentially rely on measurement of antibody binding site occupancy by analyte following reaction between the two (ref. 2). However, antibody occupancy may by determined either by *direct* measurement of occupied sites, or by their *indirect* measurement, i.e., by the measurment of *unoccupied* sites. The optimal amounts of concentration of antibody yielding maximal assay sensitivity largely depends on which of these alternative strategies is adopted, tending to infinity in the first case, and to zero in the second. (Note: this divergence is a consequence of the differing statistical implications of experimental errors inevitably incurred in the final determination of antibody occupancy. Were the measurements underlying the occupancy estimate to be entirely error-free, no antibody concentration would be optimal.) This difference in optimal antibody concentrations underlies the frequent subdivision of binding assays into "non-competitive" and "competitive" methods, albeit these terms derive historically from the (controversial) portrayal of certain assay methodologies (e.g., RIA and "competitive protein binding assays" [CPBA]) as dependent on a "competition" between radiolabelled and unlabelled analyte for a limited number of antibody (or specific protein) binding sites. The latter concept is clearly untenable in the case of assays relying on the use of labelled antibodies or the newer "immunosensor" technologies now under development (in which neither analyte nor antibody is "labelled" in a conventional sense), and in which the notion of a "competition" between labelled and unlabelled molecular species therefore does not apply. Nevertheless, in both these situations, the optimal amount of antibody critically depends on whether the signal emanating from occupied or unoccupied binding sites is measured, emphasizing that the distinction between "competitive" and "non-competitive" assays is unrelated to which, if either, of the principal reagents in the system is labelled.

Unoccupied antibody binding sites can be measured in a variety of ways, e.g., by back-titration with (radio)labelled analyte or (radio)labelled anti-idiotypic antibody, or (in the case of labelled antibody methods) by adsorption of unoccupied antibody onto a solid immunoadsorbant, followed by measurement of the immunoadsorbed labelled antibody. Meanwhile all radio-isotopically-based "non-competitive" methods rely on the use of labelled antibody. Occupied sites may likewise be identified by the use of an immunoadsorbant, though in this case labelled antibody remaining in the supernatant must be measured directly. Alternatively, a second ("capture") antibody directed against a second, unobscured, binding site on the analyte molecule may be employed to sequester analyte bound to the labelled antibody, thus creating a "sandwich", or "two-site" immunoassay (ref. 3). The sequence in which the various reagents are introduced into incubation tubes in these various immunoassay formats may also differ, causing detailed differences in assay design, and in the relative performance of the various procedures in regard to sensitivity, precision, specificity, speed, etc. For example, in the case of competitive immunoassays relying on the use of radiolabelled analyte, the optimal amount of radiolabelled analyte tends to zero if the labelled material is introduced simultaneously with the sample, but to infinity if it is added following completion of the reaction between unlabelled analyte and antibody. The latter strategy yields assays of somewhat higher sensitivity,but with a liability to assay "drift" due to the lack of thermodynamic equilibrium in the assay system.

Performance characteristics of radioisotopically-based immunoassays

The variety of assay designs makes difficult a general assessment of the performance characteristics of isotopically-based immunoassay methods. However, it is theoretically demonstrable that the maximal sensitivity attainable using a competitive method is ϵ/K, where $\epsilon = cv$ in the measurement of the response variable (e.g., fraction of labelled analyte

bound) and K = the (effective) affinity constant of the antibody (or binding protein) on which the assay relies (ref. 4). For example, assuming $\epsilon = 0.01$ (i.e., 1%) and $K = 10^{12}$ L/M (the maximum antibody affinity seen in practice), the maximal sensitivity yielded by an optimised RIA procedure will closely approach 10^{-14} mol/L, i.e., approximately 10^7 molecules/ml. These theoretical predictions are supported by experimental experience, no reported RIA ever having surpassed this sensitivity (other than by prior extraction and concentration of the analyte). Similar theoretical analysis reveals that "non-competitive" immunoassay designs are potentially considerably more sensitive, and likely to display both a wider working range and faster kinetics than "competitive" designs by virtue of the higher concentrations of antibody on which they implicitly rely. However, their greater *potential* sensitivity cannot be realized using radioisotopic labels in consequence of the limited specific activities of the radioisotopes (such as ^{125}I) commonly used in this context (since the minimal number of ^{125}I-labelled antibody molecules measurable in a reasonable counting time also lies in the range of 10^6-10^7 molecules). Thus, though non-competitive radioisotopic methodologies are generally more sensitive (particularly when only antibodies of relatively low affinity are available), both competitive and noncompetitive approaches are limited in sensitivity to analyte concentrations of the order of 10^6-10^7 molecules/ml. In order to increase assay sensitivity beyond these limits, antibody labels possessing higher specific activities than radioisotopes (such as enzymes, chemiluminescent and fluorescent substances) must be employed in assays of non-competitive design. This constitutes one of the underlying reasons for the progressive abandonment of the radioisotopic methods now taking place (see below).

In consequence of their general reliance on relatively elevated antibody concentrations, non-competitive methods are intrinsically less specific, since the potency of any cross-reacting antigen present in a test sample relative to the potency of the desired analyte approaches unity as the relative amount of antibody present in the system increases, notwithstanding the (likely) possession by such a cross reactant of a lower affinity with respect to the antibody used. This disadvantage can, however, be overcome by the use of the sandwich or two-site approach referred to above, implying that assays of this type combine the advantages of both extremely high specificity and sensitivity, albeit the very highest sensitivity is only attainable using non-isotopic labels as previously discussed.

Illustrative of these general concepts are sandwich methods for cholera toxin, rotavirus and other antigens relying on enzyme-labelled antibodies, the enzymes catalysing reactions based on fluorogenic ("HS-ELISA") and radioactive (USERIA) substrates. In both cases, the enzyme acts as an amplifier, each enzyme molecule generating many molecules of fluorescent or radiolabelled product, thereby greatly increasing the effective specific activity of the labelled antibody employed in the system. Sensitivities claimed for thee methods are in the order of 10^5 and 10^3 molecules/ml, respectively. It should be emphasized that the high sensitivities attained by the use of amplification systems of this kind could not be realistically achieved using competitive assay formats.

Costs

The extent and diversity of the immunoassay field likewise makes difficult any realistic assessment of the costs of establishing or performing assays of this kind. Commercial immunoassay kits and/or bulk reagents for a wide range of analytes are now available, and there is therefore generally little need to incur the capital costs of reagent production and assay development. For the more frequently-used assays (such as that of the thyroid hormone thyroxine) the cost of immunoassay kits (containing *ca.* 100 assay tubes) may fall to as low as 20-30 cents (U.S.) per tube, but, in the case of more rarely required assays involving more exotic reagents, the cost may be up to 10-100 fold greater. Likewise, instrumental costs vary widely, depending ont he level of automation and data reduction facilities built into the system. Typically, a multi-sample γ-counter, complete with desk-top computer for data-analysis, costs in the order of $15,000 (U.S.), but simpler single-sample manual equipment without data-reduction facilities is at least ten-fold cheaper. Other ancillary equipment that might typically be required would include a centrifuge and high precision pipetting equipment, although many technologies have now been devised which avoid centrifugal separation of reaction products. Many manufacturers have

developed completely automatic immunoassay systems which embrace sample preparation and incubation steps in addition to signal measurement, and this clearly represents an increasing trend in the field.

Future developments

"Binding assay" methodology of one form or another is likely to retain a dominant position in the microanalytical armamentarium for the foreseeable future. However, two major trends have emerged in this area since the first radioisotopically-based binding assays were described in 1960 (refs. 5, 6) and these are likely to continue. The first centers on the refinement of the means of production of the binding molecules used in this context. Initially binding assays were based on "naturally-occurring" proteins (such as serum binding proteins or antibodies produced in laboratory animals in response to immunological challenge). Subsequently, the development of the *in vitro* hybridoma techniques of monoclonal antibody production by Milstein and Köhler removed much of the element of chance associated with conventional antibody production methods, and increased control over the nature, properties, and long-term availability of the binding reagent. More recently a further step has been taken along this path by the introduction of "single domain antibodies (dAbs)" by Ward *et al.* (ref. 7) which rely on genetic engineering techniques for the *in vitro* production of the heavy chain variable (VH) domain of selected antibody molecules. Though lacking the light chain component of the specific antigen binding-site, many VH domains display binding affinities not greatly reduced as compared with the original antibody. Future predictable developments along these lines include the modification of such binding sites to increase their specificity and affinity for the analyte molecule of interest, leading ultimately to the synthesis of entirely artificial analyte-recognition binding molecules.

The second development centers on the labels employed to monitor the reactions between analyte and the "analyte-recognition reagent". Radioisotopic labels are relatively easily measurable and unaffected by their chemical or physical environment; moreover counting equipment *per se* is now widely available, familiar, and inexpensive. These features underlie the continuing popularity of isotopically-based methods. However, a number of reasons underlie the current development of non-isotopic immunoassay technologies, which represents the principal area of methodological development in the immunoassay field during the past five years. These reasons may be conveniently grouped under four main headings:

i. Non-scientific (e.g., environmental, economic, legal, logistic, etc.).
This heading reflects a variety of "non-scientific" advantages of non-isotopic methodologies, including, for example, avoidance of the problems of radioisotope disposal, the possibility of development of "non-separation" or "homogenous" methods (i.e., methods obviating the inconvenience of physical separation of the immunological reaction products prior to signal measurement), or of methods yielding coloured reaction products, avoiding the necessity for complex electronic signal-measurement equipment, etc.

ii. The demand for "ultra-sensitive" immunoassay methodologies (see above).

iii. The development of "immunosensors".
Immunosensors comprise solid "probes" or "sensors" coated with specific antibody whose reaction with analyte in the test medium is transduced into a measurable electronic or optical signal. Transduction methods and devices currently under study rely on measurement of surface plasmon resonance, evanescent wave detection, field-effect transistors, piezo-electric and surface acoustic wave devices, etc. Ideally, immunosensors would respond to changing analyte concentrations in the surrounding medium; however, the slow dissociation kinetics of high affinity antigen-antibody reactions present a major obstacle to the attainment of this objective. Other problems associated with immunosensor development are a lack of sensitivity, and a reliance on single analyte-binding-site recognition, implying a lack of specificity. At best, therefore, such devices appear likely to offer only additional convenience for high-concentration analyte measurements in special situations, e.g., as bedside tests in hospital wards.

iv. The development of multianalyte immunoassay methods.
Developments under this heading reflect an increasing need to measure many different substances in the same sample, both in medicine and in many

biologically-related fields, e.g., the food industry. The possibility of developing such technologies emerges as a consequence of the availability of very high specific activity, non-isotopic, reagent labels, e.g., fluorescent labels. This permits the development of "microspot" immunoassays in which a "sensor" or "capture" antibody is located on the surface of a probe within an area no more than 50-100 μm^2 (ref. 8). This in turn permits the construction of multi-microspot arrays, each microspot being directed against a different analyte. Technology of this kind currently under development relies on fluorescent labels, and laser scanning techniques. Such technology, closely comparable to that employed in domestic "compact disk" recordings, is likely to make a major impact on the binding assay field within the foreseeable future.

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V. POSITRON EMISSION TOMOGRAPHY

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Abstract - Positron emission tomography (PET) is a non-invasive tracer technique for application in humans. Since its development, the acceptance of this technique has grown due to the fact that it makes available a vast array of investigative measurements not possible earlier. Central to PET's uniqueness is its sensitivity and its ability to map within an organ the distributed biochemical and physiological alterations that result from a pathological, behavioral or pharmacological perturbation.

Introduction

If one describes disease as a clinical manifestation of a process initiated by an external insult of chemical origin (e.g., carcinogens, virus) its identification (diagnosis) or evolution (prognosis) using non-invasive biochemical probes become highly desirable. Positron emission tomography (PET) is an analytical imaging technique that was developed to fill that void in medicine by allowing the *in vivo*, non-invasive assessment of local, specific biochemical events in humans (ref. 1). Since biochemical changes always precede anatomical alterations (e.g., observable by conventional diagnostic techniques), the information provided by PET increases our understanding of the evolution of human disease and provides information for more specific and improved therapies.

At present, most of our knowledge of cell biochemistry has been derived from the invasive application of radioactive (e.g., ^{14}C, ^{3}H, ^{32}P) and stable (^{2}H, ^{13}C, ^{15}N) isotopes. The analytical methodologies involved; i.e., ^{15}N, ^{13}C, by mass spectrometry (ref. 2), nuclear magnetic resonance (refs. 3, 4) and liquid scintillation counting with ^{14}C and ^{3}H, have serious limitations for applications to humans. It should be noted, however, that carbon-13 and phosphorus-31 NMR have shown applicability to metabolic analyses in living

organisms (i.e., bacteria), cells and isolated organ preparations (ref. 5), but application of NMR to large intact animals is essentially restricted to ^{1}HNMR imaging. This modality, which provides excellent anatomical delineation, offers limited information in the evaluation of metabolic events (ref. 6).

Basic concepts

Quantitative information of biochemical parameters with PET is obtained by using a positron tomograph to collect data obtained by administration of positron-emitting labeled tracers into humans. By definition, all radionuclides used with PET decay by positron emission. A positron is an antielectron that upon emission from a decaying nucleus, travels a short distance before combining with an electron from the surroundings. On annihilation, two 511 keV photons are emitted at an angle of approximately 180° from each other. These photons are detected by external detectors with coincidence circuits. The data collected by annihilation coincidence detection are used to reconstruct mathematically the cross-sectional distribution of tissue radioactivity concentration into tomographic images (ref. 7). With the aid of tracer kinetic mathematical models, quantitative information can be obtained (ref. 9).

PET permits, therefore, the quantitative estimation *in vivo* of biochemical and physiological parameters, namely, tissue perfusion, membrane transport, metabolic and biosynthesis rates (i.e., neurotransmitter synthesis fluxes), and receptor affinity and density. This is possible because of major developments in radiopharmaceutical chemistry, particularly in the last decade, when hundreds of biochemically and pharmacologically active compounds were labeled with cyclotron-produced positron-emitting-radionuclides of natural elements [i.e., ^{11}C (20.4 min half-life), ^{13}N (9.96 min), ^{15}O (2.03 min), ^{18}F (109.7)] (ref. 8). Obviously, substitution of ^{11}C, ^{13}N, and ^{15}O for the natural isotopes of carbon, nitrogen, and oxygen, respectively, renders compounds biochemically indistinguishable from their natural counterparts. On the other hand, fluorine-18 is frequently used to provide labeled substrate analogs (e.g., 2-deoxy-2-[^{18}F]fluoro-D-glucose) and pharmacological agents (e.g., ^{18}F-labeled neuroleptic drugs; 6-[^{18}F]fluoro-L-dopa) that trace biochemical processes in a predictable manner (refs. 7, 9).

Assessing biochemical parameters in humans non-invasively

The list of available positron emitting labeled tracers includes radiolabeled carbohydrates, free fatty acids, amino acids, and a variety of pharmacological agents, most of them for use in either brain or heart (ref. 7). The availability of radiotracers is, however, a necessary, but insufficient condition for performing specific assay measurements. Analytical studies of biochemical processes with PET are meaningful only when the radiolabeled compounds can be accurately modeled *in vivo* using tracer kinetic principles (refs. 7, 10). Tracer kinetic models are, therefore, a necessary and major component of PET. The positron tomograph only measures local time-dependent radiolabeled concentration changes throughout the organ (i.e., brain). For conversion of these images into local reaction rates, PET measurements must be combined with the time course of radiotracer in blood and integrated with validated tracer kinetic models of the process under investigation (see for example, ref. 10).

The identification of procedures for different types of assays using PET requires the fulfillment of various biochemical and kinetic prerequisites. For the selected method to be applicable, radioactivity in tissue must be confined, at all times during the experiment, to one product or, at the most, a few well-defined chemical species. *In vivo* determination of glucose metabolic rates is a process that can be modeled following these principles. In this approach, initially applied to brain autoradiography in rodents, glucose metabolic rates are measured using [^{14}C]2-deoxy-D-glucose which ensures that the radioactivity in tissue, is contained in the deoxysugar precursor or in the product of the hexokinase reaction, 2-deoxy-D-glucose 6-phosphate (ref. 11). 2-Deoxy-D-glucose competes with glucose for its facilitated membrane transport, and subsequent phosphorylation by hexokinase. While glucose 6-phosphate is normally metabolized via the glycolytic pathway, 2-deoxy-D-glucose 6-phosphate is not a substrate for further metabolism. Consequently, its accumulation is proportional to the rate of the hexokinase mediated reaction in tissue and, under steady state conditions, to the glucose metabolic rate. A lumped constant takes into account the relative kinetic differences for transport and phosphorylation between glucose and 2-deoxy-D-glucose.

The general principle discussed above, introduced by Sokoloff and coworkers (ref. 12), was extended fourteen years ago to the non-invasive determination of cerebral glucose metabolic rates with PET, using the positron emitting labeled glucose analog, [^{18}F]2-deoxy-2-fluoro-D-glucose ([^{18}F]FDG) (ref. 13), found to retain the transport and enzymatic characteristics of 2-deoxy-D-glucose (refs. 14-17). Since PET kinetics with [^{18}F]FDG are functional (not anatomical) representations of brain activity, they proved sensitive to ambient test conditions. In resting states and during different degrees of sensory deprivation, cerebral glucose metabolic rates in normal subjects showed left-right asymmetries (ref. 18). These observations were complemented with various functional activation (sensory and motor tasks) studies in normal subjects (refs. 19-22). Also a detailed description of brain energy metabolism with functional development (ref. 23) and biochemical alterations with various diseases, including Huntington's disease (ref. 24), epilepsy (ref. 25), stroke (refs. 26, 27), tumors (ref. 28), Alzheimer's disease (ref. 29) and a variety of psychiatric syndromes (refs. 1, 7) has been provided.

Another example of the kinetic approach described above for [^{18}F]FDG is the L-[1-^{11}C]leucine method to measure protein synthesis rates in humans. In the brain, changes in local protein synthesis rates can be specifically associated with such processes as development, tissue regeneration and repair in response to injury or disease, plasticity, changes induced by drugs and hormones, degenerative disorders, neoplastic lesions and possibly learning and memory. In myocardium, proteins have metabolic, structural and mechanical (contractile) functions. Local protein biosynthesis, therefore, may constitute an indication of tissue viability after injury. Except for initial kinetic studies performed in the heart (ref. 30) the method has been almost exclusively applied to the brain using autoradiography in rats (ref. 11) and extended to man with PET (refs. 31-33).

The spatial resolution of PET scanners (2.5 to 4.5 mm) is lower than that of magnetic resonance imaging (MRI) (1 to 2 mm). The excellent anatomical resolution that MRI can provide is compensated by the very high sensitivity associated with PET which permits measurements at concentrations in the picomolar range. Therefore, this high sensitivity of PET offers a powerful tool to identify neuronal sites and study the functional activity of neurotransmitter systems in living human beings (ref. 34). Neurotransmitters are substances that participate in nerve impulses, contributing to maintain brain chemistry in a delicate balance determining behavior, emotional states, cognitive effects and neuromuscular action. Using PET to study neuroreceptors in the central nervous system, appropriate positron-emitting labeled radiotracers are injected intravenously and the temporal sequence of radioactivity concentration of unbound tracer in tissue is estimated from radioactivity levels in brain areas that are free of specific binding, or from radioactivity in blood samples. Thus, specific radiotracer binding to receptors can be related to the *in vivo* accumulation of radioactivity after injection of the radiotracer. Specific labeling of receptor systems can be inferred by appropriate anatomical localization, and demonstrated by competitive blockage, drug displacement and kinetic differentiation analysis of specific vs. non-specific binding. Positron emitting labeled ligands for cholinergic (ref. 35), opiate (refs. 36, 37), benzodiazepine (refs. 38, 39) and dopamine receptors (refs. 40-44) have been developed and used in animals and humans. In humans, presynaptic neurotransmission is being investigated with 6-[^{18}F]fluorodopa (refs. 45, 46) for dopamine systems. Postsynaptic receptor interactions have been studied with radiolabeled drugs, such as [^{11}C-*methyl*]spiperone (ref. 41), [^{11}C]raclopride (ref. 44), and 3-*N*-fluoroalkylspiperone (ref. 34) for dopamine-D2 sites and [^{11}C]carfentanil (ref. 37) for opiate (mμ) sites.

Conclusion

Using positron emission tomography, *in vivo* biochemical and pharmacological data can be obtained from normal human subjects and patients with a variety of disorders. Substrates and drugs can be labeled with positron-emitters and their kinetic behavior examined *in vivo* under tracer conditions or even at concentrations (e.g., with drugs) producing pharmacological effects. Following these procedures, for example, the effect of specific pharmacological agents on behavior or symptoms can be observed and correlated with alterations in pharmacokinetics at their sites of action (ref. 44). One of the most exciting outcomes of this research is that PET is providing for the first time the unique opportunity to examine the chemical dynamics of neurotransmission in the brain of the living human, as well as the means for assessing the biochemical basis of neuropsychiatric

diseases (ref. 34). For example, PET is now being used to investigate alterations of receptor densities in humans as a result of chronic neuroleptic treatment, schizophrenia, and affective disorders (refs. 47-50). By labeling chemicals related to the neurotransmitter dopamine (e.g., 6-[^{18}F]fluoro-L-dopa), its production can be estimated in health and disease states (e.g., Parkinson's disease, behavior modifying drugs). These, along with assays for enzymes involved with the synthesis and metabolism of neurotransmitters, permit identification of specific deficits in these systems. The approach can be designed to "dissect" biochemically the system under evaluation by studying its regulatory mechanisms in health and their perturbation in disease.

The extraordinary development of PET in the last decade now permits the investigation of many cellular processes in humans. Applications of PET in research, however, requires a multidisciplinary, collegiate effort, from production of labeled precursors to human imaging. While this is occurring, a significant amount of groundwork is being established worldwide for extension of this methodology into a clinical setting in which the various components of this technology are being simplified. Pivotal to this goal is the development of integrated systems specifically designed for the routine delivery of radiopharmaceuticals and their automatic administration to patients. These and the advent of small, inexpensive particle accelerators for the production of biochemically useful short-lived isotopes is making PET a unique prognostic (and diagnostic) tool in clinical medicine.

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VI. THE USE OF PROTON BEAMS FOR THE INVESTIGATION OF THE CHEMICAL COMPOSITION OF BIOLOGICAL SYSTEMS

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Abstract - Proton beams of a few MeV are increasingly in use for the analysis of a variety of materials. Both atomic (inner-shell ionization) and nuclear processes are used to obtain information about the chemical composition of samples including trace elements. Microprobes are able to produce trace element distributions with a lateral resolution down to 1 μm.

The analytical methods

Proton beams of a few MeV can be used to study the chemical composition of a variety of materials. The protons induce inner shell vacancies, which leads to emission of characteristic X-rays (Particle Induced X-ray Emission, PIXE), or eventually nuclear reactions which leads to a variety of techniques dependent of the type of radiation detected. Examples of the latter group of methods are RBS (Rutherford Back Scattering) with detection of elastically scattered particles; NRA (Nuclear Reaction Analysis) with detection of inelastically scattered particles and PRA (Prompt Radiation Analysis) with detection of gamma-radiation.

Since the cross section for inner shell ionization exceeds the typical cross section for nuclear reactions by several orders of magnitude, PIXE is preferably used for the analysis of trace elements. The technique is reviewed by some authors (refs. 1-8) and is particularly suitable for biological work due to the absence of X-rays from the matrix elements, which are absorbed completely before they reach the detection system.

Detection of the X-rays is commonly done with an energy-dispersive system (Li-drifted Si-detectors) although some work is in progress to develop wavelength dispersive systems also for PIXE. Detection limits reported are between 0.1 and 10 μg/g, dependent of the proton energy/analyte combination.

Advantages of PIXE are its real multi-element character with a continuous function of the sensitivity with the atomic number, the precisely known underlying physics which in principle avoids systematic errors and opens possibilities for adequate corrections of matrix effects, and the very short time needed for a complete analysis (in the order of minutes). Disadvantages are its limited precision caused by counting statistics, spectrum fitting computer codes and irreproducible side-effects introduced by the beam (radiation damage leading to changes in the overall chemical composition, charge built-up) and the limited availability of ion accelerators. The precision is limited to 5% in optimal cases. The costs are high for the equipment needed (accelerator, beam line, PIXE-chamber, detection systems) but on the other hand, the short time needed per analysis reduces the costs expressed in terms of exploitation of the set up to an acceptable level.

Sample preparation includes normally freeze-drying, homogenization and mounting on a high purity backing foil before the irradiation takes place. These simple procedures are an advantage, since it is rather easy to avoid contamination.

On the low part of the periodic table there is a limitation in the elements to be analysed due to X-ray absorption. The cut off is about $11<Z<14$, dependent of the thicknesses of windows in chamber and detector. For the lighter elements, nuclear reactions are used as a complement to PIXE, especially for the elements Na, Mg, Al and F. In Table I, one can find the major applications of PIXE used for the assessment of trace element concentrations in biological material.

Table I. Some illustrative applications of PIXE in biology

Sample type	Main observations	References
Pollen tubes	Ca gradient in pollen-tubes	9
Wheat seed	The influence of Mn on hormone auxin.	9, 40
Blood and tissue	Correlation between element concentrations and diseases.	15
Animal bone	Zn concentration in the region of calcification.	12
Necrotic tissue	Changes in elemental concentration during tumor growth.	13
Lung tissue	Elemental concentrations in bronchial carcinoma.	16
Gastric nucosa	Depletion of Cu, Se, Br, Rb in samples cancerous cells.	17
Human hair	Incorporation of trace elements into hair.	18, 20, 30
Osteons of human femur	Increased Pb-levels.	21
Teeth	Influence of dowels on heavy metal concentration.	22
Human skin	The physiology of the human skin.	25, 39
Human skin	Elemental distribution over a skin section.	23
Single blood cells	Change of concentration of essential elements after Se supplementation.	26, 27, 41
Hippocampus	Distribution of Zn and Cu in the rat brain.	19, 28, 34
Liver	Diagnosis of biliary cirrhosis.	46
Eye lenses	Changes in element concentrations during cataract.	46
Rat liver	Influence of necrosis and cirrhosis on element concentrations.	47
Human brain	Changes in element concentrations during tumor growth.	47
Sweat	Change in element concentration during hyperthermia.	47
Sea algae	Season-dependent I concentrations.	47
Herbivorous copepod	Distributions of Si, Ca, Cu, Zn, and Br.	50
Mussel shells	Historical records of the elements in the shells.	50

The nuclear microprobe

A very strong point of the use of proton beams is the option to focus the beam to micrometer sizes. With this option, one is able to scan over a biological section in order to measure trace element distributions rather than bulk concentrations. The technique is normally referred to as micro-PIXE, the instrument is called a proton microprobe, although sometimes other particles are used as well (d,α..). Detection limits are basically the same as for PIXE, since there is no dependence with the size of the beam spot; on the other hand, beam currents are much lower because collimation takes place before the final focussing, leading to higher practical detection limits in reasonable measuring times. Moreover, the scanning procedure itself divides the available X-ray events over a large number of PIXELS. The quite unique feature of micro-PIXE is the combination of micrometer resolution and sufficient detection power to operate at the trace element level. On top of that, real quantitative analysis is feasible with a proton microprobe. In Table I also micro-PIXE applications for biological research are included.

In the future most research effort will be devoted to further developments of micro-PIXE in order to obtain smaller beam spots with a sufficient current density to maintain detection power. Most promising in this respect are the newly developed field emission ion sources and progress made in the construction of achromatic magnetic- and electric quadrupole lens systems. Submicron beams should be within reach in the near future. The consequence of this progress will be that subcellular measurements of trace element concentrations become possible, although one should recognize that specimen

preparation, with of course the final aim to maintain the element distribution as it was in the living material, will become increasingly difficult, not in the least due to the penetrating power of the proton beam (about 50-75 μm) which calls for very thin specimens in order to avoid unwanted information from deeper layers.

Moreover, the very high beam density will cause radiation damage of the sample to such an extent that one can expect limitations in the applicability of those beams on soft tissue. In this context it is worthwhile to mention a recently developed method which uses synchrotron radiation instead of protons for the excitation of X-rays. Attempts to focus the synchrotron radiation (in the 10-20 keV region) down to spots of micrometer size are underway. For both the proton microprobe and the synchrotron microprobe holds that the final research goal is to determine the transport mechanism and the function of the essential trace elements in living matter and also to establish the influence of toxic elements on biological material.

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VII. PERTURBED ANGULAR CORRELATION (PAC)

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Abstract - The basic physical principles of PAC are described with examples listing its use in studying stoichiometry, coordination geometry, metal binding sites, and ultramolecular motion of biomolecules. Merits, disadvantages, and prospects of development of PAC in biology are presented.

When a radiative decay takes place through a three-level system via successive emission of two photons (Fig. 1), an angular correlation generally exists between the propagation directions $\vec{k_1}$ and $\vec{k_2}$. This angular dependence is described by the correlation function $W(\theta)$ which is the relative probability of emission of two γ rays at an intersecting angle θ. For a system in field-free space, $W(\theta)$ can be expressed in the following form:

$$W(\theta)=1+\sum_{k_{even}=2}^{k_{max}} A_k P_k\ (\cos\theta) \quad (1)$$

where the coefficients A_k contain nuclear spins of the initial (I_1), the

intermediate (I), and the final (I_f) level, and the multipolarities of the first (L') and second (L) emitted γ-radiation. $P_k(\cos\theta)$ are the Legendre polynomials. In most cases only k = 2 and 4 need to be considered. Those cascade gamma transitions whose intermediate level possesses nuclear spin equal 0 or 1/2 have no γ-γ angular correlation.

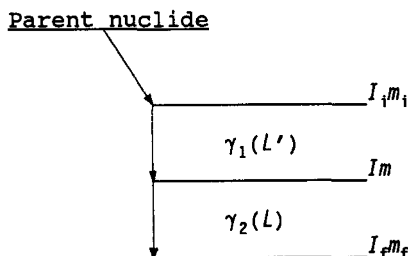

Fig. 1. Cascade gamma transition

The population of the intermediate level among the $2I+I$ substates characterized by quantum number m may be altered by the interaction of its momenta with extranuclear electromagnetic fields, resulting in a variation in angular correlation. There are two types of such interactions: (1) Coupling of the magnetic dipole moment of the intermediate level with an extranuclear magnetic field, and (2) coupling of the electric quadrupole moment of the intermediate level with an extranuclear electric field gradient (efg). Both interactions may cause transitions of the intermediate from one of its substrates to another, thus altering its population. Consequently, the angular correlation is perturbed by the fields. Theoretical analyses and experimental data showed that a time-dependent function, $G_k(t)$, describing the attenuation of the coefficient A_k under the perturbation of extranuclear fields should be incorporated into formula (1) for each A_k.

$$W(\theta,t)= \sum_{k_{even}=0}^{k_{max}} A_k G_k(t) P_k(\cos\theta) \qquad (2)$$

The attenuation coefficients $G_k(t)$ contain all the physical information about the extranuclear fields.

The perturbing fields are versatile. They may be static or dynamic, deliberately added or naturally created by chemical environment. Among them, the nuclear quadrupole interaction (NQI), i.e., the interaction of the nuclear electric quadrupole of the studied nucleus with the extranuclear efg created by its chemical environment is of most interest. Because of the inverse cubic power dependence of efg on the distance form the nucleus to the efg producers, only the nearest electrons and nuclei contribute to efg. Therefore, much knowledge about the vicinal environment of the studied nucleus may be extracted from the measured PAC spectra, such as stoichiometry, coordination number, coordination geometry, ligating atoms, binding sites, gross conformation changes, and intramolecular motion of macromolecules.

A PAC experiment requires the measurement of both the angular distribution and time dependence of coincident counts from two γ-ray detectors. A high time-resolution, fast-slow coincidence circuit is needed. Other instruments and data acquisition systems are those routinely used in modern nuclear physics laboratories. The most commonly used γ-γ cascades are from the excited states of ^{111}Cd and ^{181}Ta populated by the radioactive decays of the corresponding parent nuclides ^{111}In, ^{111m}Cd, ^{111}Ag, and ^{181}Hf. Other cascades, such as ^{99}Ru (^{99}Rh $\xrightarrow{EC}$), ^{100}Rh (^{100}Pd $\xrightarrow{EC}$), ^{187}Re (^{187}W $\xrightarrow{\beta}$), ^{188}Os (^{188}Re $\xrightarrow{\beta}$), ^{154}Gd (^{154}Eu $\xrightarrow{\beta}$) and other rare earth nuclides, are sometimes used.

The PAC method was first applied to biological problems in 1968. Since then, three main topics have been studied: (1) Coordination geometry of the metal sites in enzymes; (2) Conformation and dynamic studies of proteins and DNA; and (3) Stability and chemistry of radiolabelled monoclonal antibodies and other radiopharmaceuticals.

In PAC studies, the possible hot atom chemistry effect of nuclear decay leading to the starting level of the γ-γ cascade must be taken into account. Isomeric transitions and beta decay yield rather mild consequences, while electron capture (EC) (e.g., ^{111}In $\xrightarrow{EC}$ ^{111}Cd) leads to severe atomic and molecular perturbations. It may result in a change of the binding site in the biomolecule or a rearrangement of the electronic environment on the nanosecond time scale.

One of the advantages of PAC is that only a small amount of specimen (10^{-9}mol in 1 ml) and a low activity of radionuclide (10-100 μCi) are required. A second merit is that it is applicable in either a solid or liquid specimen and over a wide temperature range. Also, it is a non-destructive method.

However, the application of PAC is markedly constrained by these factors: (1) Limitation of the investigated biological systems due to the very limited number of appropriate nuclides (until now little has been done outside of the ^{111}Cd cascade); (2) Lack of commercially available PAC instruments; and (3) Complications in radiolabelling the biocompound of interest.

It is recommended that a wider range of suitable nuclides be considered. For instance, the toxicological study of metal pollutants by means of ^{111m}Cd or ^{199m}Hg PAC is worthwhile. In addition, several suitable rare earth nuclides could be employed in studying the interactions of rare earth elements with biological systems.

Representative application studies are listed in Table I.

TABLE I - Examples of PAC applications in biological studies.

Sample	Nuclide	Observation	Reference
Living human cell	^{111m}Cd	Cd distribution and binding sites in cultured living cell.	11
Blood platelet	^{111}In	Weak complex formed of ^{111}In-labelled platelet.	12
Carbonic anhydrase	^{111m}Cd	Structural information of Zn site in enzyme and pH dependence of catalytic activity reflected by the nuclear quadrupole interaction (NQI).	13
Protoporphyrin and apohemoglobin	^{111}In	Comparison between metallo-porphyrin's motional freedom in hemo- and myoglobin.	14
Metallothionein (MT)	^{111m}Cd	Elucidation of specific metal-binding sites in MT.	15
DNA	^{111}In	[^{111}In]histone as an intermediate probe binding to DNA. Change of DNA helix flexibility in presence of some dyes.	16
DNA	^{111}In	Comparison of In- binding sites between single-stranded DNA and double-stranded DNA.	17
Monoclonal antibody (McAb)	^{111}In	Comparison of In binding sites between In-DTPA (diethylene-triaminepenta-acetic acid) and In-DTPA-McAb.	18
Tropolone, acetylacetone	^{111}In	Transfer of ^{111}In from chelates to transferrin and hemoglobin.	19
DNA and DNA moieties	^{111}In	In binding to both phosphate and base moieties of DNA and nucleotides.	20
Blood serum	^{111}In	No distinct difference between normal and liver cancer patient's serum binding.	21
Bovine Trypsinogen	^{199m}Hg	Introducing ^{199m}Hg into the disulfide bridge of bovine trypsinogen's rigid activation domain. Its intramolecular reorientation motion over a wide angular range with a correlation time τ_c = 11 sec.	22

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VIII. MÖSSBAUER SPECTROSCOPY

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Abstract - The Mössbauer spectroscopy on ^{57}Fe is used to obtain information on the electronic structure of the iron in the active center of proteins. The conformation and the dynamics are assigned. Scattering techniques allow to study the dynamics of biomolecules which do not contain iron.

Mössbauer spectroscopy on biological systems is essentially limited to iron-containing or iron-labelled compounds. The stable Mössbauer spectroscopy isotope ^{57}Fe has an abundance of 2.2% in natural iron. Isotopical enrichment up to 95% *in vitro* or by growing cells on ^{57}Fe-enriched media increases the sensitivity of the method significantly. The Mössbauer spectroscopy allows to study the charge and the spin state of the iron with great sensitivity. Moreover, it measures the symmetry of the surrounding. From these results one obtains information concerning the number and the chemical nature of the ligands. Many investigations deal with heme proteins (refs. 1-8), iron-sulfur proteins (refs. 9-23) and other iron-containing proteins (refs. 24-27). Mössbauer spectroscopy proved to be a useful tool in studying the iron metabolism and the involved iron transfer and iron storage proteins (refs. 28-36). Determination by Mössbauer spectroscopy of the content and conformation of iron-containing proteins in the tissue may become interesting for medical applications (refs. 37-40). Of growing interest is the investigation of magnetically ordered biological material like the radular teeth of chitons (refs. 41-44).

In the last years the Mössbauer spectroscopy has become an important technique to investigate protein dynamics and motions in membranes (refs. 45-53). Examples for all types of applications are given in Table 1.

The principle of Mössbauer spectroscopy is summarized in the following: Gamma radiation emitted by the 14.4 keV level of ^{57}Fe (populated by the radioactive decay of ^{57}Co) can be absorbed resonantly by ^{57}Fe nuclei of the sample if energy losses due to recoil are avoided in both emission and absorption. In a Mössbauer spectrum the intensity transmitted through the sample under investigation is measured as function of the energy of the incoming radiation. The energy change is achieved by moving the source with a velocity v [mm/s] (Doppler effect). Therefore, the energy scale of the spectra is given in terms of Doppler velocity v [mm/s] which can be converted to eV by multiplying with 4.80768×10^{-8}. Per definition, the center of a Mössbauer spectrum of an iron foil equals $v = 0$. Usually, the energy is varied only within less than 10^{-6} eV. Due to the relatively long lifetime of the 14.4 keV level of the ^{57}Fe nucleus ($\tau_{nucleus}$ = 141 ns), the natural energy width of a Mössbauer spectrum is about 10^{-8} eV. The extreme high energy resolution is one of the merits of this method. Apart from the transmission geometry, other registration techniques using secondary radiations (e.g., Auger electrons, X-rays) play no role.

Table 1. Examples of Mössbauer spectroscopy applications in biology.

Sample	Temperature range. Remarks	Observation	Reference
Cytochrome c-554 (*Nitrosomonas europaea*)	T = 4.2 K. Magnetic field up to 6.0 T.	Spin states of the 4 hemes: at pH 2, 75% high spin; at pH > 10, 100% low spin; pH 7, 75% low spin magnetic interactions of the irons.	1
Hemerythrin	--	Reversible NO binding $Fe^{2+}Fe^{3+}NO$; Fe^{2+}: S = 2, Fe^{3+}: S = 3/2. Antiparallel coupling.	27
Putidamonooxin	T = 1.5 K - 4.2 K. Magnetic field up to 2.5 T.	Investigation of the co-factor iron (non-heme, non S-linked); binary oxidized enzyme substrate complex: Fe coordination > 4; ternary enzyme substrate NO complex: iron Fe^{3+}, S = 3/2; pentacoordinated.	10
Beef heart aconitase	T = 1.3 K, 4.2 K. Magnetic field up to 3 T.	Purple aconitase:3Fe-4S, Fe^{3+} high spin in a tetra-hedral environment of S; spin coupling, cluster spin: S = 5/2.	16
Fe protein AvZ of nitrogenase system of *Azotobacter vinelandii*	T = 4.2 K, 50 K, 150 K.	Oxidized state: $[4Fe\text{-}4S]^{2+}$ cluster; reduced state: $[4Fe\text{-}4S]^{1+}$ (S = 1/2) + $[4Fe\text{-}4S]^{1+}$ (S = 3/2).	17
Ferredoxin II from *Desulfovibrio gigas*	T = 1.3 K - 200 K. Magnetic field up to 6 T.	Fe_3S_4-cluster; reduced state: cluster S = 2; ground state $1Fe^{3+}$, two other sites: delocalized Fe^{2+}/Fe^{3+} pair. Above 20 K: population of an excited state.	20
Glutamine phosphoribosyl pyrophosphate amino-transferase of *Bacillus subtilis*	--	Investigation of 4Fe-4S clusters. Oxidation/reduction properties.	23

Table 1 (cont.)

Sample	Temperature range, Remarks	Observation	Reference
Bovine spleen purple acid phosphatase	--	Binuclear iron center bridged by a μ-oxo and additional ligands.	24
Bovine spleen purple acid phosphatase	T = 4.2 K, 77 K.	Antiparallel coupling of the high spins of two Fe^{3+} ions. Reduction transfers $1e^-$ to the iron which is less firmly bound to the protein.	25
Ferritin, haemo-siderin from human spleens	T = 1.3 K - 200 K.	Spectra typical for magnetically ordered material in small particles (super-paramagnetic behaviour). Proposal: haemosiderin is derived from ferritin.	28
Iron-marked melanosomes from bovine eyes	T = 100 K	Reduction of mobility within melanin by high water contents (structure stabilization).	37
Liver of Spitsbergen reindeer	T = 1.3 K - 77 K.	Most of iron stored as ferritin and haemosiderin. Iron content varies strongly with the time of the year.	38
Coprogen in *Neurospara crassa*	--	Iron metabolism.	31
Iron-overloaded livers	--	Spectra of ferritin and hemosiderin in addition; new molecular environment of Fe.	34
Dental enamel of sump beaver	--	The iron-containing pigment consists of high spin Fe^{3+} in small particles.	42
Teeth at different maturation stages along the radula of chitons	--	Magnetite mineralizes from a hydrous ferric oxide precursor and is later oxidized to maghemite.	43
Myoglobin crystals	T = 100 - 300 K.	Protein dynamics.	46
Myoglobin crystals, chick embryo fibroblasts, *Escherichia coli*, Mycoplasma membrane	T = 0°C; 10°C.	Brownian motions in proteins.	50
Myoglobin crystals	T = 4.2 K - 300 K.	Protein dynamics. Quasi diffusive motions of molecular segments, comparison with molecular dynamic simulations.	51
DNA; myoglobin	T = 90 K - 300 K. RSMR	Dynamics; dependence on hydration.	56
Chromatophore membranes	T = 80 K - 300 K. RSMR	Dynamics of membranes.	57
Myoglobin crystals	T = 300 K. RSMR	Protein dynamics, size of collectively moving segments.	58

Figure 1 shows as an example the very simple Mössbauer spectrum of deoxymyoglobin. The splitting of the absorption lines (quadrupole splitting QS) reflects the low symmetry of the iron environment (4N of the domed protoporphyrin and 1N of histidine). The shift of the center of the absorption area (isomer shift IS) indicates (together with QS) Fe^{2+} high spin. The area $A^{(0)}$ of the spectrum measures the probability of recoil free processes. Knowing the probability for recoil free emission in the source, $A^{(0)}$ becomes proportional to $\exp(-k^2\langle x^2\rangle)$ where $k = 2\pi/\lambda$ and $\lambda = 0.86$ Å for ^{57}Fe. In the absorber (sample under investigation) the mean square displacement, $\langle x^2\rangle$, of the iron depends on the chemical bonds of iron to its neighbours, the stability of the three-dimensional structure and the aggregation. Measuring the temperature dependence in a wide range (4.2 K to 300 K) allows to separate the different contributions and to give information on the protein specific part of dynamics. The life time of the 14.4 keV level introduces a time threshold. Only processes which are faster than about 100 ns contribute to the $\langle x^2\rangle$ value. As seen in Figure 1, the two Lorentzians (solid line) do not fit the experimental data. The additional broad quasielastic absorption lines indicate the presence of diffusive motions of molecular segments.

In comparison to other spectroscopic techniques like EPR or NMR, Mössbauer spectrometry is rather inexpensive. Results are very precise and can normally be compared quantitatively with theory since the influence of Fe electrons on the nucleus via hyperfine interactions is well known. The limitation to iron compounds should not be overestimated. Biochemical processes such as nitrogen fixation, photosynthesis (refs. 54, 55) or respiratory chain reactions involve a large number of iron proteins. Moreover, for the investigation of dynamics, some systems can be labelled with ^{57}Fe. Using scattering techniques (Rayleigh Scattering of Mössbauer Radiation, abbreviated RSMR), the sample under investigation has not to contain a Mössbauer isotope (refs. 56-59). In these experiments, the radiation from a Mössbauer ^{57}Fe source is scattered like x-rays by the electrons of the sample (Rayleigh Scattering). A Mössbauer absorber (e.g., stainless steel) brought into the scattered beam allows an energy analysis. Only elastically scattered quanta can be absorbed by the Mössbauer effect. From the ratio of elastic to total scattering, mean square displacements of all atoms of the sample are obtained with the time resolution of Mössbauer spectroscopy. Radiation of other Mössbauer isotopes will be used here in the future, too.

In the years 1983-1988, several review articles have been written on protein dynamics (refs. 45, 52, 60, 61). Summarizing articles have been written on ferredoxin by Johnson (ref. 62) and on hemoglobin by Parak and Trautwein (ref. 63). More general reviews come from Dickson (ref. 64) and Maeda (ref. 65). To get a fast overview on publications after 1988, one can read the contributions to Mössbauer spectroscopy on biological systems at the ICAME 1989 in Hyperfine Interactions (1990) 58, 2329-2417.

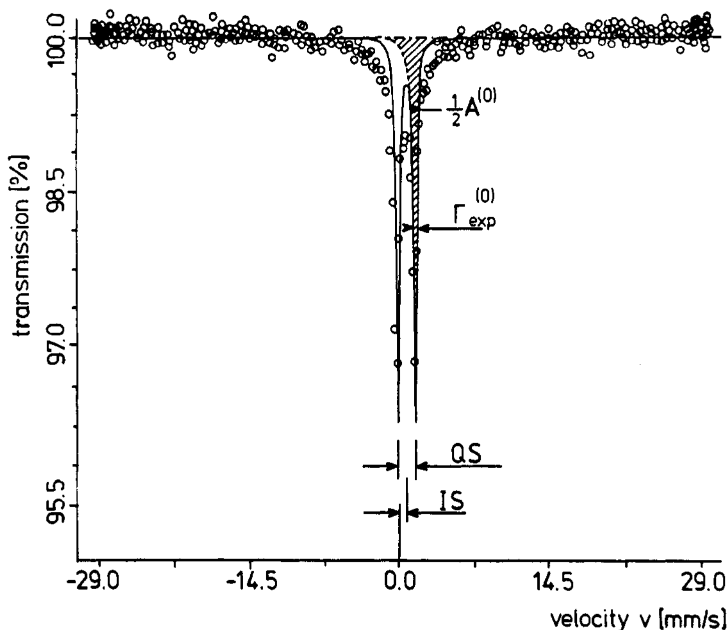

Figure 1.
Mössbauer spectrum of deoxymyoglobin. See text for details.

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