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IODINE-LABELED PLASMA PROTEINS

Volume II
Part A

Erwin Regoeczi

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PREFACE

The first volume of the present monograph is technologically oriented, and therefore its approach is mainly analytic. In contrast, the emphasis in these concluding volumes (Volume II, Parts A and B) is on information synthesis. An attempt is made to put labeled protein molecules in perspective with respect to a variety of applications both in vivo and in vitro. Likewise, radioactive products of protein degradation are viewed in the context of related metabolites, such as iodide and thyroid hormones. Laboratory and clinical work involving labeled proteins cannot be rewarding unless the function and structure of a protein, as well as the possible impact of iodine substitution on both, are considered together. In my view, these three aspects (i.e., structure, function, and labeling) are as inseparable in modern biology as were form and substance to the thinking of Aristotelian metaphysicians.

Like the first volume, Volume II was also written primarily with the uninitiated in mind, whose time would be saved by having the scattered literature in a collated form at his/her disposal. However, the amount of information published in relation to the subject area in recent years is overwhelming, whereby the idea of providing a comprehensive coverage of the field within the space made available by the Publisher had to be abandoned right at the beginning. Nevertheless, it is hoped that the way selected topics are presented will provide some guidance for the exploration, by interested individuals, of any protein tracer not described herein.

Volume II includes a short chapter on counters and counting. Events like the following provided an incentive for its inclusion. One day, a young colleague came asking for help because the gamma spectrometer he was using had apparently broken down. On inspection, the instrument turned out to be in perfect order, except that the analog-to-digital converter was jamming as the result of a huge count overload. Few young investigators know nowadays how a counter operates. It has become customary to rely on, and believe in, fancy print-outs. This is understandable. The frontier of science, like the front line on several occasions during the North Africa campaign[1,2] of World War II, now lies hundreds of miles away from the Mediterranean ports of disembarkation. Young graduates are, just as Montgomery's Eighth Army and Rommel's Afrika Korps once had been, compelled to march a long way before they can fire a meaningful shot. And along the march route are to be found, among other things, counters.

I am much indebted to the Medical Research Council of Canada for continuing career and personal financial support without which this endeavor would not have been possible. I am also indebted to Dr. J. C. Laidlaw, Dean emeritus of McMaster University, for his support and encouragement, and to CRC Press, Inc., for the editorial work. Last, but not least, I wish to thank sincerely my wife and family, colleagues, associates, and friends for their (gradually dissipating!) patience during the months of my self-ostracism.

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Dr. Regoeczi has authored or coauthored over 100 papers in various journals mainly on the use of labeled proteins, and he is a member of several learned societies at home and abroad.

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Chapter 1

DISTINGUISHABLE BEHAVIOR OF IODOPROTEINS AND THEIR DETECTION

I. INTRODUCTION

As discussed in some detail in Chapter 3 of Volume I, there are numerous ways of substituting radioiodine into proteins. We also considered later in the same volume (p. 118) a simple technique, namely, acid precipitation, by which the outcome of the labeling reaction can quickly be assessed with respect to proper substitution. Assuming this test yields a satisfactory answer, the product is, per se, ready to be used. It is this point from which the present chapter takes its departure to draw potential users' attention to the absolute necessity of qualifying the properties of all radioiodinated proteins as tracers before embarking on full-scale experimentation.

A. The Tracer Concept as Applied to Plasma Proteins

The concept of a tracer, biological and biochemical alike, entails that the behavior of a tagged molecule be indistinguishable from that of its untagged counterpart. The sole justification for creating an "artificial" molecule is the desire to learn more about *natural* molecules.

There are many aspects to a plasma protein from an investigational point of view. Fibrinogen, for example, may be studied as a subject of protein synthesis and degradation; as a protein that possesses binding sites for other proteins, such as plasminogen, plasminogen activators, and α_2-antiplasmin; as a substrate for the proteolytic enzymes thrombin and plasmin; as a macromolecule that exhibits a tendency to adsorb to surfaces at interfaces; as a potential antigen after its administration to another species; and so on. Clearly, one might be asking for too much by requesting that radioiodinated fibrinogen should be indistinguishable from the native protein in all aspects mentioned above. There may exist universal tracer proteins which are true, unconditional substitutes for the native molecules whatever the purpose of a study might be, but they are probably few and far between. It is a commonsense practice, therefore, to restrict the tracer concept to a particular application in mind.

B. A Frequent Logical Error in Establishing the Applicability of the Tracer Principle

On the basis of the foregoing it seems a reasonable expectation that any investigator who deploys a radioiodinated protein in a specific experimental arrangement would ascertain that, in the context of the prevailing technical design, the marker functions as a proper tracer. Gratifyingly for readers of many research papers, this is, indeed, often the case.

Nevertheless, publications still appear not infrequently in which assessment of the suitability of an iodoprotein as a tracer has been made using inappropriate premises. Statements, like "the labeled fibrinogen electrophoresed indistinguishably from native fibrinogen, and it was fully precipitable with monospecific antibodies raised to the native protein", are pertinent tracer qualifications only if the labeled fibrinogen is going to be used as an electrophoretic or an immunological tracer of the native molecule. They are rather irrelevant, however, if the labeled protein was prepared for an in-vivo study, because the electrophoretic mobility, antigenic determinants, and catabolic rate of fibrinogen are three independent aspects of the same polypeptide structure. Consequently, the demonstration that substitution of iodine did not affect two of these aspects does not justify the inference that the third aspect of the native state has been preserved as well. Subjecting labeled proteins to general tests of the kinds referred to in the above example may facilitate the detection of gross

alterations. However, failing to find any is by no means a guarantee of tracer-like behavior as far as many potential fields of application are concerned. The golden rule therefore is that the tracer quality of a labeled protein should be verified, whenever possible, directly in relation to its intended usage.

C. Scope of the Problem

There are numerous known cases of iodoproteins "malfunctioning", i.e., not reflecting truthfully the normal performance of the corresponding native proteins under a given set of experimental conditions. Deviations from the normal can occur in either direction. Thus, an intrinsic property of the native molecule may become amplified, while another is diminished (and sometimes even rendered nonexistent) as the result of labeling.

The aim of the present chapter is to highlight some of the more common ways in which labeled proteins may behave distinguishably and to suggest possible means of detecting such situations.

II. THE OVERALL STRATEGY

A prerequisite to the successful identification of the reason for disappointing results with any iodoprotein is the realization that the problem can be quite variable, depending on the involvement of one of the factors listed. A labeled protein may behave abnormally because (1) it has been denatured independently of the labeling procedure, and/or (2) it has been altered during or as the result of labeling.

Detailed discussion of the causes, forms, and detection of protein denaturation is beyond the scope of the present treatise. As a general comment, it is worth, however, pointing out that the more frequent sources of denatured proteins are

1a. The use of aged or inappropriately stored starting material (blood, plasma, etc.) for plasma protein purification.
1b. Harsh protein isolation procedures.
1c. Storage for too long or under inappropriate conditions of a purified protein fraction destined for subsequent labeling.
1d. Radiation damage incurred on storing a labeled preparation.

Some of these issues will be dealt with in detail in Chapter 5, Sections I.C, II.B.2, and III.B. Meanwhile, the relevant point here is that any of the above sources are more or less readily identifiable from the results obtained after changing the experimental protocol, preferably by one variable at a time. If the problem persists after reisolating the protein from fresh plasma by another (milder) technique and using it without delay, the chances are that

2a. The damage is brought about by a chemical reaction which accompanies the main reaction that leads to labeling.
2b. The atom(s) or group(s) introduced in the molecule are incompatible with the envisaged tracer function.

The two situations are not identical. As may be perceived from the following examples, malfunctions belonging to category 2a result from side reactions and *not* from the presence of the label that had been incorporated. Tetrachloroglycoluril (Iodogen) can be used to catalyze oxidation of iodide to iodine;[1] if the reagent is present in excess, or the reaction is allowed to continue beyond optimal time, transferrin[2] and albumin (see Volume I, p. 53) contained in the reaction mixture undergo an oxidative side reaction which manifests itself in tighter sorption to an anion-exchange resin. The whole anomalous binding is reversible up to a point by subsequent reduction, and the phenomenon can be reproduced with prelabeled

proteins (and in the absence of iodide), thus demonstrating that iodotyrosyl residues are not involved. Two methionyl residues in human α_1-proteinase inhibitor (Met-351 and Met-358) are particularly sensitive to oxidation by the chloro-compound, *N*-chlorosuccinimide,[3] or by myeloperoxidase.[4] Oxidation of these residues considerably weakens the inhibitor's association with proteases,[5] suggesting that they are of critical importance for the reactive center configuration.[6,7] It is easily seen that because of this particular predisposition to oxidative damages, iodination of α_1-proteinase inhibitor by using an oxidizing technique, such as chloramine-T, could readily effect inactivation of the reactive center by a mechanism which is distinct from the substitution of the halogen per se. (Labeling of α_1-proteinase inhibitor by the chloramine-T method for metabolic studies has been accomplished before, but the inhibitory properties of the product are not known.[8])

As to malfunctions of the category-2b type, here the diminished or lost tracer function is directly related to the presence of an exogenous element or group introduced in the native molecule as a label. Apart from the steric hindrances which may be created by conjugating radioactive aromatic structures, altered ionization of substituted tyrosines and loss of chargeable groups consumed in coupling reactions can change the net charge of a protein so as to make it functionally discernible from its native counterpart. Furthermore, conjugated aromatic structures may contribute to polypeptide hydrophobicity, and substitutions in or near active and reactive center configurations, as in arginine kinase,[9] may suspend enzymic and inhibitory activities. Chapters 3 and 5 of Volume I list many examples which substantiate these arguments.

Plasma proteins, being large structures, contain numerous ionizable groups, whereby a subtle change, as after labeling on a limited scale, is often "balanced out" with no untoward effects in many areas of application. This is a distinct advantage of plasma proteins over small polypeptide hormones. Category 2b malfunctions lend themselves to correction by changing the labeling method to another one that relies on introducing the label at a different site in the protein. Consultation of the literature in this regard should be done in a guarded way: perhaps because of the brevity requested by the editors of many journals, few investigators specify their labeled product beyond naming the method they used to obtain it. However, as is already familiar from Chapter 3 of Volume I, most labelings can be deployed over such a wide range of reaction conditions as to render the products hardly comparable. This is certainly an area where economizing on space does not pay.

III. MANIFESTATIONS OF IODOPROTEIN MALFUNCTIONS

In this section different fields of application are described in which iodoproteins have been known to yield, regularly or sporadically, results that are not representative of the native proteins. The emphasis will be on problems in relation to category 2b. It follows from Section I.A that labeled proteins which malfunction in one particular aspect can still behave normally when deployed in another field of study.

A. Metabolic Studies on Living Beings

Measurement of catabolism of iodine-labeled proteins is the most widely used approach to establishing the turnover rate of plasma proteins in health and disease. Conclusions from such studies can be misleading if:

1. The protein does not tolerate labeling with iodine at all.
2. It does, but not in the quantities used in a particular instance.
3. The labeled preparation is metabolically heterogenous.

1. The Suitability of a Protein to Be Labeled with Iodine

It is probably true to say that there are few plasma proteins, if any, which could not be

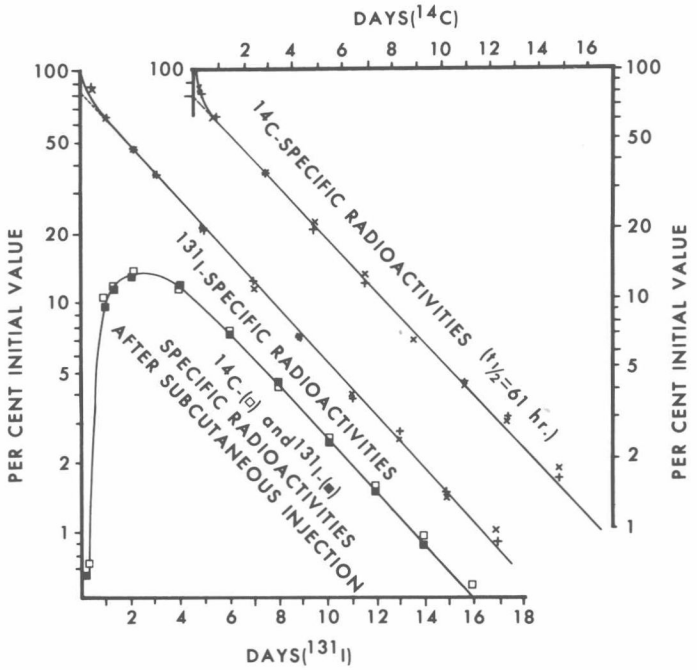

FIGURE 1. Behavior in vivo of simultaneously injected ^{14}C- and ^{131}I-fibrinogens. Two rabbits (denoted as "+" and "x", respectively) received a mixture of ^{14}C-biosynthetic and ^{131}I-fibrinogens of homologous origin on day 0. The ^{131}I-preparation was screened (see Section III.3) for 24 hr before mixing with the ^{14}C-protein. The plot of specific activities was obtained by withdrawing small blood samples at the times indicated on the abscissa and measuring radioactivities in fibrin. Also shown are the plasma radioactivity curves of the same preparations after injection into multiple subcutaneous sites in a third rabbit. Note that the reference (^{14}C) and test (^{131}I) proteins were catabolized at the same rate. (From McFarlane, A. S., *J. Clin. Invest.*, 42, 346, 1963. With permission.)

labeled with iodine for metabolic studies when the attempt is made by the right technique under optimal conditions. One should take this attitude in practice until all avenues that are available through today's wide range of technology are exhausted.

The ultimate proof of behavioral identity of a labeled protein with its native counterpart is furnished in dual-isotope experiments, performed in the same recipient and at the same time (Figure 1). The test protein is of course the one that carries the iodine label. The reference protein must be a preparation with no extraneous matter attached to or incorporated in the polypeptide. There are two groups of techniques for obtaining such reference proteins. In one of them, the protein is labeled biosynthetically, i.e., through the incorporation in vivo of radioactive precursor amino acids.

a. ^{14}C Reference Proteins

A mixture of uniformly labeled amino acids can be prepared by hydrolysis of the proteins from the green alga *Chorella,* grown in the presence of ^{14}C. (Both the algal protein and its hydrolysate are available commercially at specific activities in excess of 50 mCi/mg atom carbon, e.g., from ICN Radiochemicals, Irvine, Calif.) The [^{14}C]fibrinogen depicted in Figure 1 was obtained by giving algal protein hydrolysate to a rabbit by stomach tube and exsanguinating the animal 5.5 hr later for the isolation of fibrinogen. The test ^{131}I-fibrinogen had been injected in the same animal earlier for simultaneous reisolation with the biosynthetically labeled protein, a measure aimed at the equalization of any damage that might be afflicted on fibrinogen during purification.

b. ^{75}Se Reference Proteins

Another way of producing biosynthetically labeled reference proteins is by injecting [^{75}Se] methionine (selenomethionine; [^{75}Se]Met). ([^{75}Se]Selenoproteins can also be obtained[10] by keeping rats on drinking water supplemented with a mixture of [^{75}Se]selenious acid and unlabeled selenite; this approach is the preferred way of labeling tissue proteins under equilibrium conditions, while for plasma proteins it seems unnecessarily time consuming.) The isotope decays by electron capture (100%) with a half-life of 120 days and emits several rays. The energies of the principal radiations are 136 and 265 KeV, and those of the minor ones 121, 280, and 401 KeV.[11] This analogue of Met is produced when baker's yeast *(Saccharomyces cerevisiae)* is grown on a medium containing $H_2{}^{75}SeO_3$ and insufficient sulfur.[12] (Selenium cannot entirely replace sulfur for the growth of *Escherichia coli* but [Se]Met can serve as the sole source of Met.[13]) It is available commercially in radiopharmaceutical quality, e.g., from E. R. Squibb and Sons, Inc. Its disappearance from the plasma is rapid, less than 1% of the dose being detected in human blood free 1 hr after i.v. injection.[14] Sometimes it is administered intraperitoneally.[15]

Tissues which most actively acquire [^{75}Se]Met in the rat are the pancreas, intestine, liver, and kidney.[16] Of the blood components, both cells[17-23] and plasma proteins[16,19-21,24,25] become labeled. Erythrocytes incorporate approximately 4% of the dose in man, and over 85% of the red cell activity is associated with hemoglobin.[26] ^{75}Se activity peaks in plasma proteins approximately 8 hr after injection in man,[14] and after 5 to 6 hr in rats[15,25] and rabbits.[27] It has been calculated that at this time, in man, 20% of the dose is in the intravascular plasma protein pool.[14] [^{75}Se]Met, unlike radioiodide, is retained in the body for considerable periods of time. Thus, according to a study in two humans, 13% of the dose is lost with a half-life of less than 1 day, 44% with one of 46 days, and 42% with 220 days.[28] Radioiodide (^{131}I) can contain a trace ($<0.02\%$) of ^{75}Se, probably as selenite, and so after the administration of a large therapeutic dose (thyroid carcinoma), the body may contain detectable amounts of residual ^{75}Se activity.[29]

^{75}Se administered as selenite disappears rapidly from the human circulation, but a portion of it reappears 1 to 6 hr later largely bound to VLDL and LDL. This association is initially loose, i.e., it is reversible by denaturation with 8 *M* urea or treatment with 0.5 *M* mercaptoethanol. However, the same treatments remove very little of the ^{75}Se from plasma collected 48 hr after injection indicating a change in the type of binding of selenium in lipoproteins.[907]

In view of the definition of reference proteins given above, it seems legitimate to ask whether [^{75}Se]Met is fully comparable with ^{14}C-labeled amino acids as a biosynthetic label. The answer is best attempted by juxtaposing [Se]Met to [S]Met. It seems firmly established that selenoamino acids (selenomethionine and selenocysteine) are natural body constituents. Selenocysteine lyase, an enzyme that specifically decomposes the selenoamino acid into alanine and H_2Se, has recently been isolated from the pig liver.[908] [Se]Cys constitutes the catalytic center of several redox enzymes,[30,31] notably that of glutathione peroxidase.[10,32-34] Rat liver synthesizes [Se]Cys from [Se]Met through selenohomocysteine in the analogous sequence to the sulfur-containing counterparts.[35] [^{75}Se]Met is transported across everted hamster intestinal sacs at the same rate as [^{35}S]Met in the presence of carrier methionine.[36] Furthermore, [^{75}Se]Met is reutilized to about the same extent as [^{35}S]Met.[36]

In view of these similarities, one would expect that [^{75}Se]Met becomes incorporated into plasma proteins with similar kinetics to [^{35}S]Met. In contrast to this expectation, Herrman,[381] and Motsenbocker and Tappel[15] found that, 3 hr after injecting it in rats, 73% of the ^{75}Se analogue was still present in a circulating protein of 45 to 50 kDa which was thought to be its transport protein. Such discrepancies may arise because the utilization of [Se]Met is possibly determined by the nutritional state of the recipient with respect to sulfur. When dietary sulfur amino acids are plentiful, [Se]Met might be more readily catabolized than in the opposite case and the liberated Se made available for the synthesis of genuine selenoproteins, such as glutathione peroxidase. (Amino acids supplied in excess are degraded.)