The Synthesis of Proteins—A Simple Experiment To Show the Procedures and Problems of Using Radioisotopes in Biochemical Studies

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The distinction between organic chemistry and biochemistry is very unclear; most people regard the former as merging into the latter. For this reason, and for reasons of student interest and future employment, very many organic chemistry courses contain studies on biochemical processes including metabolic pathways, the associated reaction mechanisms, and relevant techniques.

One of the main aims of the simple experiment described here is to illustrate the crucially important point that radioisotopes can be used to follow metabolic pathways and gain vital information on their nature and operation. The synthesis of protein is one of the most fundamental and interesting of biological processes and obviously it is desirable to be able to demonstrate it, and, if possible, to examine some of the factors affecting it. However, while experiments can be devised involving isolated ribosomes, mRNA, and so on, the lability of these materials and the time required for their preparation are awkward. The cost of some essential chemical components for such experiments can also be off-putting.

In the exercise described here the biological aspects of the experiment are simple. This not only helps with the problems noted above but also allows the teacher to focus on the second and more important aim, which is to show students unfamiliar with the techniques the procedures and difficulties involved in using radioisotopes. The unique problems of analyzing the data and the extreme sensitivity of the techniques are also brought out. The use of plant material overcomes the emotive, financial, and legal problems associated with animal work, and experience indicates that even students on courses dealing with animal and medical aspects of biology readily accept this. There is no reason why other biological material could not be used if this were more appropriate.

Plants freely take up organic molecules from solutions and can use these metabolically. The experiment therefore consists initially of incubating a young plant or seedling with a radioactive amino acid. From a homogenate, proteins can be isolated by solvent precipitation, and reassurance that they very probably are radioactive can be obtained by a precipitate washing technique. A series of samples taken during the successive procedures shows the change in activity as the various extraction and purification processes are undertaken. The stages of the experiment are displayed pictorially in Figure 1.

Virtually any convenient soft-tissued species of plant can be used successfully and it is probable that most amino acids would be suitable for the experiment. Tritiated alanine (L-[2,3-^{3}H]alanine from Amersham International Plc, UK) is ideal, since tritiated amino acids have the advantages of cheapness and not producing a radioactive gas by-product. However, they do require a scintillation counter for their estimation. Geiger counting of a ^{14}C-labeled amino acid and its products is too inefficient to be recommended. Furthermore, suitable precautions for dealing with $^{14}CO_2$ output from the plant would be needed.

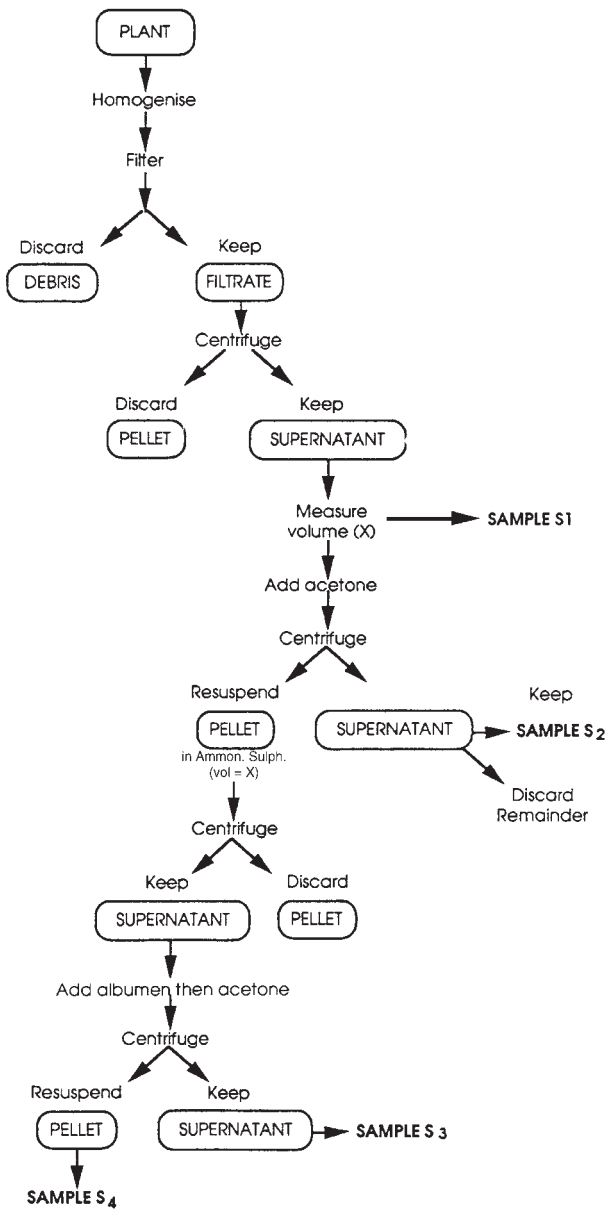

Figure 1. Extraction of protein.

Of course, some preliminary instruction on safety procedures is necessary. Several comprehensive lists of rules (*1*) and guides to safe procedure (*2*) are available in the literature and from suppliers such as Amersham Plc and NEN. Additionally, students should be advised that common sense in this as in other areas of laboratory practice is one of their best safeguards. The relative simplicity of the experimental manipulations will help students cope with the special procedures involved in isotope work. A list of the most important safety precautions is given later in this report, and this could perhaps be copied, used as a basis for discussion, and given to the students.

Experimental Work

Plant material is required for this experiment. Seedlings can be grown in ordinary gardening compost or, better, in vermiculite (mica) flakes. However, most of the small, ordinary, soft-leaved house or garden plants such as pelargoniums, primulas, or coleus can be used and these are easily purchased. It is important to safeguard as much root as possible while preparing the plant, yet to wash off most of the growing medium. Bacteria colonizing the medium can take up the amino acid as well as the plant roots and it is desirable to minimize this process.

Place the roots of the washed plant or seedling into a 100-mL conical flask with 50 mL of a nutrient solution (such as 1% casein hydrolysate) and 100 kBq (2–3 μCi) of a tritiated amino acid. Incubate overnight (or even over the weekend) at room temperature with illumination. Remove the plant and place it in a large beaker. To displace surface-bound labeled alanine, wash the roots with 100 mL of water, followed by 50 mL of 1% L-alanine solution. Place the plant over a small beaker and use scissors to cut it into several pieces.

Transfer the pieces to the cup of a small homogenizer and add a small volume (e.g., 30 mL) of water. If the homogenizer cup does not have a lid, then seal any opening at the top of the cup with film before homogenizing the tissue pieces. If a large bottom-driven homogenizer is to be used it may be necessary to pool several plants for homogenization. This may also be done for advance preparation (see the end of this section). It is helpful to keep the volume of water quite low, since acetone will be added in proportion to this, and the volume can multiply to awkward levels.

Filter the homogenate (containing all plant materials) through a small plug of glass wool or, preferably, via a perforated Buchner funnel into a conical flask and centrifuge at full speed on a bench centrifuge for 3–5 min to remove small tissue and cell fragments. Use a cap or otherwise cover the centrifuge tube.

Measure the volume of the supernatant (which is now free of debris) and transfer 1 mL to a numbered scintillation vial (S1). Use a measuring cylinder to add two volumes of acetone to the remaining supernatant and then allow the solution to stand for 10 min. If the precipitate is heavy and settles, decant and discard some supernatant before centrifuging the rest for 5 min. Remove the supernatant (which has lost most of its protein) and transfer 1 mL of it to the next scintillation vial (S2). Discard the remaining supernatant.

Use a glass rod and a hand homogenizer to resuspend the protein precipitate in a volume of very dilute (0.005%) ammonium sulfate (to help dissolution by "salting in") equivalent to that of the original supernatant. Centrifuge the suspension at full speed for 3 min to remove particulate material (including denatured protein) and again measure the volume of the supernatant. Add 1 mL of 3% albumin as a bulk carrier and mix gently. Add two volumes of acetone and allow to stand for 10 min.

Centrifuge for 5 min, remove the supernatant (which again has had protein removed), and transfer 1 mL to a scintillation vial (S3). Resuspend the final protein precipitate in 2 mL of ammonium sulfate and then transfer 1 mL to a scintillation vial (S4).

Add 1 mL of water to the 5th scintillation vial (S5). This will be used to determine background radioactivity. Then add 5 mL of a scintillant capable of accepting aqueous samples to each of the five vials.

The above experiment takes 2–2.5 hours from the end of the overnight incubation, plus the time required to measure radioactivity. However, tutors might like to carry out the homogenization, filtration, and first centrifugation themselves. This will further decrease the hazard involved, the homogenate can be stored frozen if necessary, and the rest of the experiment then only takes 1.5 hours. There are two precipitation stages that take 10 min but can be extended, and which are therefore suitable periods for discussion or rest breaks.

Results

The following discussion assumes that samples have been measured on a reasonably complex scintillation counter. The instrument will determine the radioactivity of each of the vials and the results will appear on a data printout. Modern scintillation counters are capable not only of measuring count number and calculating count rate, but also of subdividing these into radioactive emissions of different energy levels. Among the results will therefore be the count rate in two energy channels (or windows), preselected by the manufacturer for that isotope, and also a ratio of these two values. This can be used in correcting the results to eliminate a particular source of error.

The problem is that the radioactive count rate produced by the machine is always less than the true disintegration rate because of inherent and variable inefficiency in the scintillation production and counting processes—principally the phenomenon of quenching. Colors in the samples, various dissolved compounds (including oxygen), suspended particles, and writing on the vial walls (!) all contribute to reduced count rate. In this experiment, chlorophyll and acetone are present and quench significantly. This problem is virtually unique to this analytical method, and since its extent will almost certainly be variable it must be corrected for before the results can be interpreted. Variable quenching renders a direct comparison of the results printed by the instrument meaningless. Another problem unique to radioisotope work, the effects of radioactive decay during the counting process, will not be important here due to the long half-life of tritium.

Quench correction can be carried out in several ways. With a modern scintillation counter the sample channels ratio technique is straightforward. This makes use of the fact that when quenching occurs some disintegration events are not seen by the counter; but more importantly, the average energy level tends to fall. Thus the count profile tends to move from the higher energy window into the lower; the channel ratio will therefore change. A calibration curve of channel ratio against counting efficiency can be constructed using standards

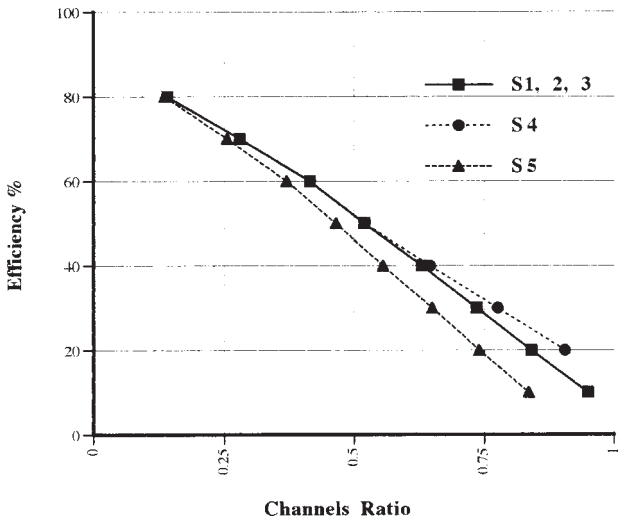

Figure 2. Quench correction graphs.

Table 1. Counting Efficiency and Channels Ratio[a]

Efficiency (%)	S1 CR	S2 CR	S3 CR	S4 CR	S5 CR
10	0.950	0.950	0.950	—	0.835
20	0.840	0.840	0.840	0.905	0.740
30	0.735	0.735	0.735	0.775	0.650
40	0.630	0.630	0.630	0.645	0.555
50	0.520	0.520	0.520	0.520	0.465
60	0.415	0.415	0.415	0.415	0.370
70	0.280	0.280	0.280	0.280	0.255
80	0.140	0.140	0.140	0.140	0.135

[a]KEY: S1-S5 are the experimental samples. CR is the channel ratio value.

of known activity quenched to various extents with different volumes of the samples to be measured. Ideally the curves should be produced at the time of the experiment using the samples generated in the experiment, as is the case with any other calibration curve. However, for convenience, a specimen is given in Figure 2 that can be used if desired. The data are supplied as Table 1 for more accurate construction by the reader.

Analysis

As mentioned above, experimental data will be printed from two measurement channels. It is important that radioactive events are not allowed to contribute twice to the final results. Therefore the data must not be automatically summed, since in some measurement programs the channel energies actually overlap. In the absence of knowledge of the energy distributions it is best to use the channel producing the higher count rate for data analysis.

To do this all the high-channel results should be corrected by using their channel ratio value and the graph to determine counting efficiency and then correcting to 100% efficiency. True disintegration rates will then be obtained for comparison. Students can then be asked to discuss the meaning of the results in the light of their knowledge of the properties of proteins and of the experimental procedures employed. Typically the homogenate (S1) has the highest disintegration rate because it contains free alanine, radioactive protein, and other metabolites of the alanine. S2 and S3 have lower activities because they are supernatants and washings of the protein precipitate. S4 generally has a higher activity than S3, but the actual values are not important because this is not intended to be a quantitative experiment. While the procedures employed do not prove the proteins to be radioactive, it is likely that they are, and the experiment could always be extended if desired by using electrophoresis or other purification procedures.

Additional Experiments and Developments

A useful extension of the exercise with regard to emphasizing the benefits of radioisotopes in chemistry and biochemistry is to calculate the amount of material actually present in radioactive form. While this analysis is very much simplified and therefore not absolutely accurate, the very low figure produced can impress on students the extremely low detection limits of isotope work. Students will have determined the true disintegration rate for S1 along with the other samples. Given that 1 Bq of radioactivity produces one disintegration per second (1 Ci gives 3.7×10^{10} dps) and given a value for the specific activity of the amino-acid starting material (10^{15} Bq mol^{-1} is fairly typical for the Amersham product) they should calculate the quantity of radioactive material in S1. Values of 10^{-13} to 10^{-15} mol are usual.

Students could also be asked to discuss briefly the advantages and problems involved in using radioisotopes in biological experiments. Support for this part of the exercise could come from refs *3*–*7* and of course from details of protein synthesis and relevant aspects of plant physiology in standard texts on these subjects, if the class tutors wish to develop this aspect further.

Potential experimental extensions are numerous. Proof that the final precipitate contains protein could be obtained from positive results with standard protein assays such as the biuret, Lowry, or Coomassie reaction described in texts on laboratory biochemistry. Rigorous proof of the synthesis of radioactive protein would require separation of the proteins, and electrophoresis techniques, perhaps coupled with a demonstration of autoradiography, could be employed. Hydrolytic cleavage of the protein and chromatographic isolation of radioactive amino acids would be possible. A combination of some or all of these extensions would enable the experiment to be expanded into a small project suitable for advanced students of biochemistry, organic chemistry, or analytical methodology.

Safety

The normal quantities of the radioisotopes used in most biological experiments do not present a great hazard to health. The β radiation of these isotopes is fairly weak and is readily absorbed in liquids and solids.

The situation in which radioisotopes are particularly dangerous is when they are absorbed by the body following ingestion. After absorption they are generally distributed throughout the body and will be intimately

mixed with materials that are sensitive to radiation damage—for example, nucleic acids, proteins, and cell membranes. A second danger comes from their decay processes. Carbon-14, for instance decays to ^{14}N, and if the ingested ^{14}C is used in the biosynthesis of cellular components, there will be a continuous degradation of these ^{14}C atoms to nitrogen. This will introduce dramatic changes in the chemical and biological properties of the compounds.

It is essential therefore that absorption of isotopes be prevented and contamination of the environment reduced to its lowest level. To avoid these dangers the rules listed below should always be observed when using radioactive isotopes; but bear in mind that such a list cannot be exhaustive nor cover every situation. Students must develop an attitude that ensures that every action is thought about beforehand and procedures are carried out carefully. These rules relate to the experiments in this paper and are not comprehensive (they do not cover use of γ-emitters or labeled gases, for instance).

Tutors and students may find it easier in practice for one member of each pair of students to carry out all operations directly involving the radioisotopes and the other member to act as a general assistant.

Rules to Reduce Body Contamination and Ingestion

1. **Laboratory coats and rubber or disposable gloves and safety goggles should be worn at all times.**
2. **Eating and drinking are forbidden in the laboratory. Use disposable paper handkerchiefs if required.**
3. **All wounds below the wrist, even if covered with plasters or bandages, provide points through which isotopes can easily enter the body. Such wounds should be reported to the class supervisor, who will decide if it is safe to handle isotopic material.**
4. **Mouth operations of all kinds (especially pipetting) are strictly forbidden in the laboratory. Do not lick adhesive labels, or insert anything else into the mouth, or apply cosmetics.**
5. **If radioactive solutions are spilled on the skin, wash well with soapy water and report to the class supervisor.**
6. **On leaving the laboratory, wash your hands and monitor your clothing for contamination by using a suitable hand monitor.**

Rules to Reduce Environmental Contamination

It is essential to avoid contaminating the environment, particularly that of a laboratory where radioactive materials are used. Careless work could, over a period of time, result in accumulation of isotope to produce high background count-rates, contamination of glassware, or dangerous radiation levels.

7. **No unnecessary materials, paper, books, clothes, or other articles should be taken into the laboratory or placed on the benches.**
8. **All work should be carried out on surfaces that are easily cleaned, e.g., plastic laminates or plastic trays. These should be covered with absorbent paper, which is treated as contaminated waste at the end of each experiment.**
9. **Any spillage occurring during the experiment should be mopped up with absorbent paper, the area sluiced with water or solvent, and mopped again. The paper is then treated as contaminated waste. Inform the class supervisor.**
10. **It is imperative that contamination of the counting apparatus be avoided. Only correctly prepared samples should be taken into the counting room, and rubber gloves should be removed before entering the room.**
11. **At the conclusion of the experiment, pour all active liquids into clearly labeled bottles kept for this purpose. Never pour waste down the sink. Radioactive solid waste should be placed in marked pedal bins or polyethylene bags.**
12. **Ideally, glassware and apparatus used with radioisotopes should be well washed and kept separate from nonactive materials. If there is a likelihood of active organic materials adhering to the apparatus it should be washed in chromic acid solution.**

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