

LIQUID SCINTILLATION COUNTING OF THE ISOTOPE Fe⁵⁵*

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INTRODUCTION

Isotopes of iron in medical investigation—The importance of iron as an essential element in many biological systems has been recognized for years. For example, iron is present as an essential component in hemoglobin and in certain widely distributed enzyme systems, (peroxidase, the cytochromes, catalase, etc.). The development of radioisotopes of this element led to great strides in the field of iron metabolism, particularly with respect to iron turnover, rate of hemoglobin formation, and distribution of iron within the body. Studies of these phenomena have contributed greatly to an understanding of iron activity in normal physiological processes and in altered mechanisms in disease. Much of this work has been done with the use of the single isotope Fe⁵⁹, an easily detected gamma emitter.

In 1946, PEACOCK *et al.*¹ introduced the simultaneous use of Fe⁵⁹ and Fe⁵⁵ in biological studies, and in particular, applied the method to the determination of red cell mass. SAYLOR and FINCH,² in 1952, extended the use of the technique, making possible the precise determination of iron absorption in human subjects. BOTHWELL *et al.*³ recently applied this method to the determination of the relative absorption of different compounds of iron. It is certain that double label methods will provide a powerful tool for the investigation of aberrations of iron distribution and storage, both of which present complex hematological problems.

Current method—Peacock's procedure¹ has been the only satisfactory method for the determination of Fe⁵⁵ and Fe⁵⁹ in a single sample. His method, in brief, is as follows. The sample of blood or tissue is digested, and the iron separated by precipitation as the hydroxide, redissolved in acid, quantitatively electroplated on planchets, and subsequently counted with differential Geiger tubes. Peacock developed G-M tubes one of which was sensitive to Fe⁵⁵, and the other to Fe⁵⁹ with less than 3% crossover between the two. However, in practice, the efficiency of counting of Fe⁵⁵ by this method is only

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of the order of 0.5–1.5%, requires the time consuming and tedious procedure of quantitative electroplating, and must be counted with specialized equipment.

Liquid scintillation technique—On speculative grounds, three potential advantages of a liquid system seemed evident: (1) simplification of technique, (2) greater counting efficiency, and (3) rapidly increasing availability of liquid counting apparatus. A completely satisfactory method has not yet been developed, and this conference paper is presented as a progress report.

EXPERIMENTAL

Instrumentation—The Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, La Grange, Illinois) was used for all measurements. The temperature of the refrigeration unit was maintained at + 8°C. Samples for counting were contained in 20 ml low-activity glass counting vials, (Wheaton Glass Company, Millville, New Jersey).

The physical problem—The average beta-particle energy of Fe⁵⁹ is 0.12 MeV. One component (54%) has a maximum energy of 0.46 MeV and the other (46%), a maximum energy of 0.27 MeV. Associated with these are gamma radiations of 1.1 and 1.3 MeV. Fe⁵⁵ emits an X ray of maximum energy of 0.0059 MeV.⁴ It was noted above that most biological applications of Fe⁵⁵ as a tracer would be in methods requiring double labeling. The challenge, therefore, is one of simultaneously counting the two iron isotopes, the one a potent beta-particle emitter, and the other, an extremely weak X-ray source.

In order to explore the possibility of differentiating the two isotopes, the following preliminary experiment was performed. A volume of 0.002 ml of a very high specific activity aqueous solution of radioactive iron sulfate was added to 2 ml of absolute ethanol. The volume was brought to 20 ml with toluene counting mixture. The resulting 10% ethanol in toluene produces minimal quenching and will easily hold the minute amount of water and iron salt in homogeneous mixture at the + 8°C temperature of the counting chamber. The toluene scintillation mixture contained PPO (diphenyloxazole) 4 g, and POPOP 1,4-bis-2 (5 phenyloxozolyl)-benzene 0.050 g per l. of toluene (LSP-2 and LSP-7, Tracerlab Inc., Boston, Mass.).

A window of 10–70 V was arbitrarily selected. The efficiency of count of each isotope was then determined at a series of photomultiplier voltages. The results are presented in Fig. 1. It is evident that under these conditions excellent separation of the isotopes was achieved. Fe⁵⁹ reached a peak efficiency of approximately 50% at a photomultiplier voltage of 940 V and fell off rapidly above this level. In the case of Fe⁵⁵, no activity was observed at the 940 V level, and the peak efficiency of about 8% was found at 1300 V. At the latter photomultiplier voltage level, the efficiency of counting of Fe⁵⁹ was of the order of 10%. It is evident, therefore, that Fe⁵⁹ can be determined in a mixture without interference from Fe⁵⁵. The crossover of Fe⁵⁹ to the

Fe^{55} peak was 20% of the Fe^{59} maximum. Therefore, if the dose of each isotope were adjusted to result in equal counts in the sample, the crossover counts of Fe^{59} at the 1300 V level would be of the order of 20% of the true

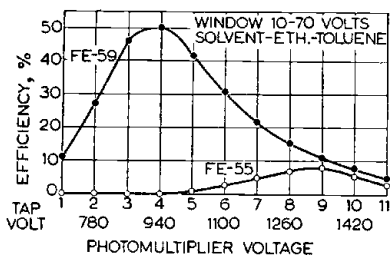


Fig. 1. Efficiency curves for counting Fe^{59} and Fe^{55} by liquid scintillation techniques. Abscissa: photomultiplier voltage. The tap settings are arbitrary voltage taps on the particular counter used in these experiments. Ordinate: counting efficiency expressed as counts observed divided by calculated disintegrations per min $\times 100$.

Fe^{55} count. This percentage is small enough so that with reasonable accuracy of determination of crossover, the error in Fe^{55} measurement will be small.

The chemical problem—Biological limitations, particularly in human studies where it is desirable to use as little tracer as possible, impose severe conditions. The ratio of total iron to tracer is very high in biological specimens, particularly in blood. This requires the incorporation of a large amount of total iron in the counting solution. Therefore, the iron compound must be colorless, non-quenching, and soluble in the organic solvents used in liquid counting.

In a typical study in human patients, administration of a safe dose of radioactive iron results in low activity in a blood sample. At a counting efficiency of from 2 to 5%, 10 ml of red blood cells would be required to obtain a practical level of activity, and the digestate of this volume contains 10 mg of iron, an amount great enough to preclude the use of a colored compound. It should be noted that if greater counting efficiency were achieved, one should reduce the amount of isotope administered rather than reduce the size of the analytical sample. It can be assumed, therefore, that about 10 mg of elemental iron must be used for analysis.

The first attempt was to prepare the iron compound of 2-ethylhexanoic acid, commonly referred to as 'octoic acid' ('2-ethylhexoic acid'—Carbide and Carbon Chemical Co., New York, N.Y.). Drs. J. R. Arnold and J. Shedlovsky of Princeton University (personal communication) have prepared manganous octoate and have found that this compound is highly soluble in toluene and produces very little quenching. The iron compound of octoic acid is intensely red in color. Reduction to the ferrous form before preparation of the octoate was not successful even in a nitrogen atmosphere. Although colorless at first, the compound rapidly reverted to a deep red color. It is

possible, however, that appropriate conditions for the formation of this compound will be developed in the future. Similar experience was observed with versenates and citrates.

It seemed most practical to prepare the iron salt of a strong acid, and subsequently convert this with reducing agents to the colorless ferrous form. Ascorbic acid was found to be a satisfactory reducing agent in this system. The chloride and nitrate were found to be somewhat unstable. The sulfate, and toluene-sulfonate were quite stable but were not soluble enough in toluene-ethanol-water mixtures. At the present writing, the trichloroacetate appears to be relatively satisfactory but preliminary studies suggest the possible superiority of perchlorate salts of iron.

Solvent system—Suspensions, dioxane-water mixtures, and toluene-ethanol-water mixtures were considered.

Suspensions were found to be difficult to prepare and very variable with respect to counting efficiency when the compound was a salt of Fe^{55} . It would be expected that the weak emission of Fe^{55} would require extremely small and constant particle size.

Although dioxane is highly miscible with salt-water solutions, three disadvantages of this compound as a solvent resulted in its elimination: (1) instability of count, (2) rapid formation of colored iron compounds and (3) chemical luminescence on exposure to light. The last objection can be eliminated by working in a darkroom. Repeated chemical purification of the dioxane would undoubtedly correct the first two objections to some extent but would increase the technical problems.

Toluene-ethanol-water mixtures were finally adopted for routine study.

Present method—The following method is currently in use. The precipitated ferric hydroxide containing 10 mg of elemental iron is dissolved in 0.5 ml of 8 N trichloroacetic acid. After the precipitate dissolves, the tube is heated in a boiling-water bath for one hour. At this point, the solution has been concentrated to a volume of less than 0.5 ml and much of the trichloroacetic acid has distilled off. Small flakes of ferric hydroxide adhering to the walls of the tube are washed to the bottom during this process. The addition of 20 mg of ascorbic acid to the concentrated red solution immediately results in the formation of a crystal-clear water-white solution. Care must be exercised at several points. Too much ascorbic acid results in an inky black solution. If the sample is evaporated too far and too little water is left, clearing will be slow or may not occur. The solution is then taken up in 3 ml of absolute ethanol and dissolved in 12 ml toluene counting solution, prepared as described in a previous section. Removal of oxygen was not ordinarily performed.

Calibration—Since some quenching occurred, calibration presented a major problem. An attempt was made to equalize the degree of quenching by standardizing the handling of both samples and prepared standards.

The internal standard procedure conventionally used with variable quenching systems was found to be unsatisfactory. The simple opening of a sample vial to introduce an internal standard resulted in an unpredictable loss of counts of the order of 10–20%. Previous air or nitrogen equilibration of the solutions resulted in minimal improvement. Some experiments were performed using a special counting vial. This vial resembled the conventional vial except that the top was drawn out into a narrow neck, which exposed but a very small surface of the liquid to the atmosphere. The calibrating solution was introduced with a thin pipette inserted through the neck of the vial. Mixing was achieved by the introduction of a small stirring rod which would effectively mix the solution in the vial without disturbing the small volume of the solution in the neck which was in contact with the atmosphere. This procedure eliminated the previously observed loss of counts due to opening the vial. Although the modified method could provide an adequate way of internal calibration, it is entirely too cumbersome for use as a practical procedure.

Results—Solutions prepared as described were found to be quite stable. Some have been preserved at $+8^{\circ}\text{C}$ for as long as a week without significant change in count. However, considerable quenching was observed, resulting in a decrease in the efficiency of counting Fe^{55} to approximately 1.5%. Furthermore, the quenching effect resulted in a shift of the Fe^{59} peak to a photomultiplier voltage tap very close to the Fe^{55} peak. This introduced a marked potential error since a high Fe^{59} count at the Fe^{55} voltage tap requires high precision in the determination of crossover. In spite of the difficulties noted above, Fe^{59} can be determined with a precision of the order of $\pm 5\%$ and Fe^{55} with a precision of about $\pm 10\%$. An occasional sample, however, will be in error with respect to Fe^{55} by as much as 20% or more.

Current investigation—One of the major difficulties with the method as presented is evidently the variable degree of quenching. This results in decreased efficiency and complicates calibration.

Attempts to standardize the handling of the trichloroacetate have included initial precipitation as the sulfide, vacuum drying the ferrous salt, and introduction of other reducing agents. None have produced marked improvement in consistency of degree of quenching.

Recent experience with the perchlorate offers promise. Preliminary data suggest that quenching is minimal. A word of warning must be presented at this point since there is some excess perchloric acid left after the hydroxide is dissolved. The potential hazards of the addition of perchloric acid to organics are well known and our experience has not been extensive enough to warrant a statement on the safety of this procedure.

Summary—A method has been described for the counting of Fe^{55} and Fe^{59} in a single sample. Considerable work is required before a routine procedure can be recommended. It has been shown, however, that the two isotopes can

be quantitatively determined by liquid scintillation techniques at a higher level of efficiency than possible with other methods.

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REFERENCES

- ¹ W. C. PEACOCK, R. D. EVANS, J. W. IRVINE, Jr., W. N. GOOD, A. F. KIP, S. WEISS and J. G. GIBSON, The use of two radioactive isotopes of iron in tracer studies of erythrocytes. *J. Clin. Invest.* **25**, 605 (1946).
- ² L. SAYLOR and C. A. FINCH. Determination of iron absorption using two isotopes of iron. *Amer. J. Physiol.* **172**, 372 (1953).
- ³ T. H. BOTHWELL, G. PIRZIO-BIROLI and C. A. FINCH. Iron absorption. *J. Lab. Clin. Med.* **51**, 17 (1958).
- ⁴ R. LOEVINGER, Average energy of allowed beta-particle spectra. *Phys. Med. and Biology.* **1**, 330 (1957).

ADDENDUM

Since submitting this paper for publication, extensive work has demonstrated that quenching is almost negligible with iron perchlorate. Furthermore, most of the difficulties described above are eliminated by use of the perchlorate method.