III. MATERIALS AND METHODS

MATERIALS

1. Subjects:

Volunteers were selected from workers in Siriraj Hospital Medical School. These subjects consisted of 60 nurses, 60 practical nurses (females, 16-20 years old), 60 janitors, and 60 blood donors (males, 20-25 years old).

2. Reagents:

All chemicals were of analytical grade.

1). conc. sulfuric acid (Sp. gr. 1.84)

2). conc. nitric acid (Sp. gr. 1.42)

3). conc. perchloric acid (70%)

4). 0.1 and 1.2 N hydrochloric acid

5). conc. ammonium hydroxide (33 %)

6). ferric chloride, 0.18 M in 0.1 N HCl

7). ascorbic acid-HCl reagent : 40 mg ascorbic acid per milliliter of 1.2 N HCl. This solution was prepared immediately before use.

3. Scintillation Gel:

3 gm PPO, 0.3 gm POPOP, 1000 ml toluene were mixed in

a stoppered bottle by electric stirror. The solution was ready for use immediately or could be stored for up to three weeks away in darkness. The scintillator was finally prepared using triton X-100 as carrier in a ratio of 12 : 7.

4. Apparatus:

1). Kjehldahl flask, 300 ml

2). centrifuge tubes, 50 ml

3). counting vials, 20 ml, of low potassium glass, with polyethylene screw caps.

5. Equipment:

1). Liquid scintillation system (Nuclear-Chicago, model 724-725).

2). Auto-Gamma Well (Nuclear-Chicago, model 88725).

METHODS

1. Design of Experiments:

The subjects were served in four consecutive mornings two meals and two reference doses, one in each morning. The food and

Day	Food & reference dose administrations
1	Food ^{55}Fe
2	Food ^{55}Fe
3	Reference dose ^{59}Fe
4	Reference dose ^{59}Fe
14	Absorption measurements

Fig. 3. Design of the experiments.

the reference dose of ferrous ascorbate were added 7/2 μCi of Fe-55 and 3/2 μCi of Fe-59 respectively. The order of administrations was reversed in the supplementary studies, i.e. ref dose, ref dose, food, food (Fig. 3). Two weeks later, blood was drawn for measurements of per cent absorption of iron from food and also for that from the reference dose.

2. Method of Analysis:

Digestion: $FeCl_3$ solution was added to 10 ml of the whole blood sample to make the total amount of iron in the flask of approximately 10 mg , then 10 ml of H_2SO_4 and HNO_3 were added.

The mixture was heated gently in a fume hood to avoid bumping, until brown fumes of the HNO_3 ceased to be evolved and white sulphur trioxide fumes were noted. If any charred material remained, 5 ml HNO_3 was added and this step was repeated. The digest would become brown but free of solids.

2 ml of perchloric acid was added when the flask was cool and the solution was heated for 20 minutes. The hot digest would be yellowish and became colourless on cooling. If not, this step was repeated.

5 ml of water was added. The digest was reboiled briefly to get rid of traces of nitric and perchloric acids.

After the flask was cool, ammonium hydroxide was cautiously added by flowing along its sides while the digest was swirled until a permanent precipitate of ferric hydroxide was obtained (about 35 ml in volume). If the flask was allowed to stand for some minutes , crystals might appear, but they were readily redissolved by few milliliters of water.

The contents of the flask were quantitatively transferred to the centrifuge tubes by the aid of two 4 ml rinses with 0.1 N HCl.

Ammonium hydroxide was again added dropwise until heavy brown floccules appeared while the digest was swirled.

After centrifugation the supernate was discarded. Rarely , peptization of the ferric hydroxide would cause cloudiness of the supernatant. This was rectified by chilling the tubes in ice-water and the tubes were recentrifuged.

3 ml of 1.2 N HCl was added to the tubes and the precipitate dissolved. the contents were transferred to counting vials. Rinsing was repeated to ensure complete transfer.

By adding a few drops of ammonium hydroxide while stir - ring with a small glass rod the precipitate of iron would appear. The vials were centrifuged and supernate discarded.

The precipitate was dried completely in oven at 95°C for about an hour. The precipitate was yellowish in colour.

After cooling, 1 ml of the ascorbic acid-HCl reagent was added and the precipitate dissolved. Now a colourless solution should be finally obtained.

19 ml of the scintillation gel was added. Vials were capped tightly and shaken vigorously in a vertical motion for one minute. During the shaking a transient yellow colour might appear, but it should disappear in a very short time. Now the gel was clear or colourless.

The vials were placed in cooling compartment of the spectrometer which was maintained at 5-8°C and they were allowed to stand for at least 2 hours before counting.

3. Preparation of Standards and Blanks:

The standards were prepared by using measured volume of ^{55}Fe in one vial and ^{59}Fe in another, and, the activity should be equal to that of the administered dose of each subject.

The blanks were made by adding 19 ml of the scintillant gel to 1 ml of the ascorbic acid-HCl reagent.

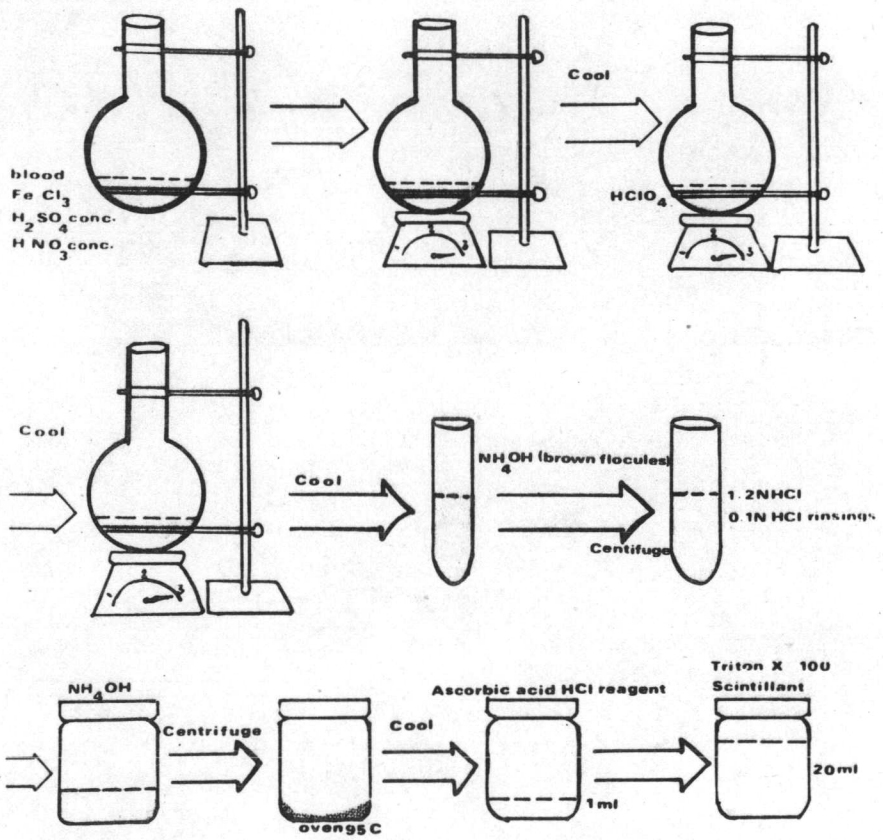

Fig. 4. Diagram illustrating steps of digestion of samples.

4. Activity Measurement:

By Auto Gamma Well Counter at the optimal operating level for ^{59}Fe , the standards, blanks, and blood samples were counted for their activities (Fig. 5). 2 ml of the blood sample was measured, without any digestion. The main purpose was to find the window width optimum for the spectrum of ^{59}Fe. From the measurement, the base has been chosen at 200 V, and window width was at 300 V.

5. Measurement by Liquid Scintillation System:

The method by which optimal channels were determined in simultaneous assay of two isotopes has been reviewed elsewhere(9,17). The Nuclear-Chicago Liquid Scintillation system 724-725 was set up to bring the spectrum of ^{55}Fe and ^{59}Fe well to the left of the gain scan as shown in Fig. (8) and (9).

It should be emphasized that all liquid scintillation spectrometers vary with regard to photomultiplier and high voltage characteristics so that the precise adjustments should have been made for any particular instrument in use. In this experiment the technique of adjusting window width and voltage (or gain) for optimal ^{59}Fe, counting at a point where ^{55}Fe is completely " silent " was employed. However, one inevitably obtained ^{59}Fe impulses at the settings necessary to achieve good counting rates for ^{55}Fe. Then the latter

setting was chosen to give a compromise between a minimal cross counting ratio for ^{59}Fe and a maximal efficiency to background ratio for ^{55}Fe. Having found the best settings, the samples and standards were counted in each channel, the cross counting ratio of ^{59}Fe (k) was accurately determined. The activity of the individual isotopes was then calculated as follows:

$$^{59}Fe \text{ counts} = (cpm)_A$$
$$^{55}Fe \text{ counts} = (cpm)_B - k\,(cpm)_A$$

where $(cpm)_A$ and $(cpm)_B$ were equal to net counts per minute at optimal ^{59}Fe and ^{55}Fe settings respectively, and k was equal to $(cpm)_B / (cpm)_A$ for ^{59}Fe.