# Fate and Distribution of $Fe^{59}$ Labelled Ferrocyanide in Humans and Dogs.* (22715)

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Previous studies(1,2,3) have demonstrated that unlabelled ferrocyanide ion fulfills many of the criteria required of a substance which would measure the volume of the extracellular fluid. After intravenous injection it equilibrates rapidly in a "space" approximating 20% of body weight; it does not penetrate or cannot be detected in red blood cells, gastric juice or feces; and from 94 to 98% of administered dose can be recovered in urine in 24 hours. In addition, Berliner and his associates(4) were unable to demonstrate by dialysis any binding of ferrocyanide to plasma proteins.

Unfortunately the quantities of non-radioactive ferrocyanide which must be used in "space" measurements were found by Miller and Winkler(5) to be nephrotoxic in humans. The present investigation was, therefore, undertaken to evaluate the fate and distribution in humans and dogs of small quantities of sodium ferrocyanide labelled with $Fe^{59}$.

*Materials and methods.* Studies *in vivo* were carried out in 9 human subjects and 3 nephrectomized dogs. The former included 4 male medical students, 2 patients with ascites and anasarca, one cirrhotic without ascites or edema and 2 patients with severe renal failure secondary to chronic pyelonephritis. In all experiments 30-50 mg of $Fe^{59}$-labelled sodium ferrocyanide (approximately 75 $\mu$c) were injected intravenously from a calibrated syringe. Measured amounts of inulin were also injected into the 3 dogs and in one subject with renal failure. Arterial samples of blood from the dogs and venous samples from the humans were collected at intervals varying from 10 minutes to one hour. Time required to reach equilibrium and the rate of disappearance of radioactivity from the blood were determined and the apparent volumes of distribution at equilibration were calculated by extrapolating back to zero time in the usual way†(1). An aliquot of the injected solution was counted simultaneously with all biologic samples, to correct for physical decay. In most studies urines were collected for 24 to 48 hours to determine the recovered radioactivity, and in addition, in 5 experiments hourly urine collections were made to determine simultaneous renal clearances of creatinine and radioactive ferrocyanide. Radioactivity in the 48-hour stool collections of 3 subjects and in the gastric juice and saliva of one subject was also measured. The distribution of radioactive ferrocyanide between plasma and red blood cells was determined *in vivo* and *in vitro* utilizing technics previously described(1). The diffusibility of radioactive and non-radioactive ferrocyanide from aqueous solutions and plasma *in vitro* was evaluated by two technics: 1) a simple dialysis procedure in which the sample to be dialyzed was placed in a bag of Visking membrane and dialyzed against a solution of isotonic saline for 24 hours with continual shaking, and 2) the anaerobic ultrafiltration technic of Lavietes(6). The *in vivo* and *in vitro* binding of radioactive ferrocyanide by specific plasma proteins was determined by electrophoresis of samples of serum on paper at pH 8.6 and 7.4 in acetate buffer (.04M) on a Spinco apparatus at 0.6-0.7 MV. Duplicate strips of each sample of serum were run simultaneously. One was stained with bromophenol blue dye to locate the distribution of

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† The Donnan factor for Fe $(CN)_6^{\equiv}$ is 1.22, *i.e.* the concentration of Fe $(CN)_6^{\equiv}$ in interstitial fluid is 1.22 × (concentration in plasma water). Since approximately 25% of extracellular water is contained in plasma at equilibrium, the "Average" concentration of Fe $(CN)_6^{\equiv}$ in extracellular fluid (plasma water + interstitial water) equals $\frac{(3 \times 1.22) + (1 \times 1.00)}{4} = 1.16 \times$ (concentration in plasma water).

the proteins, the other was cut transversely into 2 cm. pieces, and both were counted in a well-type scintillation counter. A solution of $Fe^{59}$ $(CN)_6^{\equiv}$ in isotonic saline was simultaneously electophoresed to determine the distribution on the paper of non-protein-bound ferrocyanide ion. Total proteins were precipitated from sera using 10% trichloracetic acid. All samples were counted in duplicate for one to 5 minutes depending on the level of radioactivity. The minimal counts exceeded those of the background by at least four times. The average background with the apparatus used was approximately 115 counts per minute. Plasma and transudate proteins were determined by the biuret technic(7); inulin, endogenous creatinine, and non-radioactive ferrocyanide by methods previously described(1).

*Results. Urinary recovery and renal clearance of radioactive ferrocyanide*: In the 4 normal subjects an average of 80% (68-87%) of administered radioactivity was recovered in 24 to 48 hours. This contrasted sharply with the 94 to 100% of non-radioactive Fe $(CN)_6^{\equiv}$ recovered in 24 hours when administered to normal dogs(1) and infants (3). In Table I are listed the simultaneous renal clearances of creatinine and $Fe^{59}$ $(CN)_6^{\equiv}$ in 4 normal subjects and one patient (M.S.) with severe chronic renal disease. The ferrocyanide clearance ranged from 20 to 53% of the concomitant creatinine clearance. The highest percentage was in the subject with renal disease.

*Distribution between plasma and red cells.*

TABLE I.* Simultaneous Clearances of Creatinine and $Fe^{59}$ $(CN)_6^{\equiv}$.

| Subject | Creatinine clearance, cc/min. | Ferrocyanide clearance, cc/min. |
|---|---|---|
| C.K. | 118 | 41 |
| R.S. | 130 | 48 |
| M.W. | 122 | 34 |
| M.G. | 125 | 25 |
| M.S. | 5.6 | 3.0 |

* Each value represents the mean of 4-5 consecutive periods of urine collection. Creatinine and Fe $(CN)^{\equiv}$ clearances were corrected for serum water; Fe $(CN)_6^{\equiv}$ clearance has also been corrected for a Donnan factor of 1.22.

TABLE II. Difference between Measured and Expected Concentrations* of $Fe^{59}$ $(CN)_6^{\equiv}$ in Whole Blood.

| Samples | % difference (expected-measured) |
|---|---|
| 7 human *in vivo* | $+3.6 \pm 3.9$† |
| 3 " *in vitro* | $+ .3 \pm .7$ |
| 4 dogs *in vivo* | $+1.0 \pm 2.4$ |

* "Expected concentration" in epm/cc whole blood = (plasma concentration) (1-Hct), assuming no penetration of red blood cells by radioactive ferrocyanide in the plasma.
† Stand. dev.

*Absence in gastrointestinal secretions. Table II*: As had been previously noted with non-radioactive ferrocyanide(1), there was no significant penetration of the radioactive ion into the red cell. This held true whether the tracer was added *in vitro* to whole blood at 4° and 37°, or injected intravenously into humans and dogs. In addition, when red cells, from whole blood which had been labelled with ferrocyanide, were washed with cold isotonic saline and then resuspended in saline they contained no appreciable radioactivity. No significant radioactivity was detected in pooled feces, saliva, or gastric juice of humans or dogs.

*Disappearance curves and apparent volume*

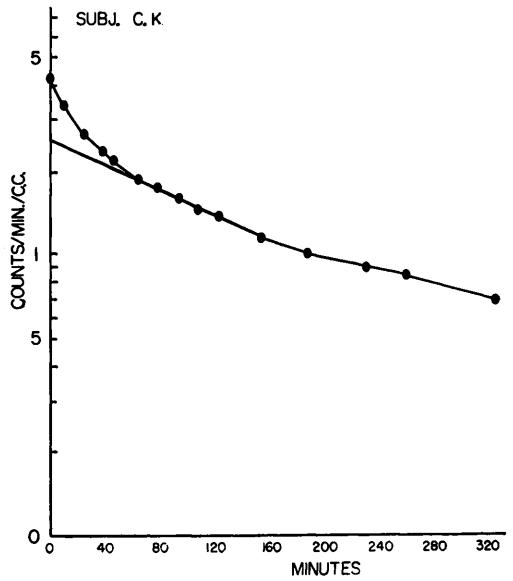


FIG. 1. Semilogarithmic disappearance of $Fe^{59}$ labelled ferrocyanide from the plasma of a normal subject.

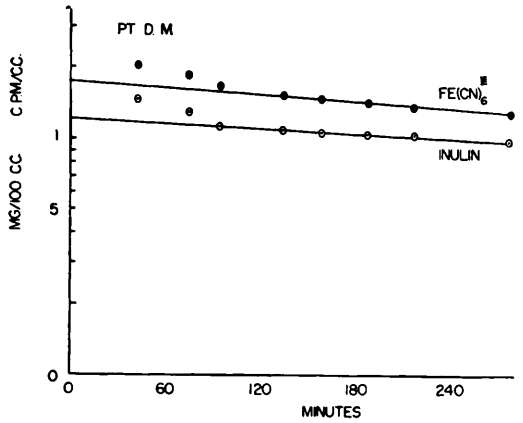


FIG. 2. Simultaneous semilogarithmic disappearance of $Fe^{59}$ labelled ferrocyanide and inulin from the plasma of a patient with severe chronic renal disease.

*of distribution in humans and nephrectomized dogs.* Fig. 1 illustrates the typical semilogarithmic curve of disappearance seen in a normal subject after apparent equilibration. The disappearance curve was linear between 60 and 160 minutes. Subsequent points fell slightly above the extrapolation of the 60-160 minute line. This lack of linearity after 160 minutes was similar to that seen in normal dogs after injection of non-radioactive ferrocyanide(1). As would be expected; the rate of disappearance of the tracer was much slower in a subject with severe chronic renal disease (Fig. 2). In contrast to normal subjects, neither patient with renal disease demonstrated a changing slope in the late portion of the curve. The similarity between the rate of disappearance of inulin and ferrocyanide is apparent (Fig. 2).

TABLE III. Apparent Volumes of Distribution of Ferrocyanide and Inulin.

| Subject | $Fe^{59}(CN)_6^{\equiv}$, % body wt | Inulin, % body wt |
|---|---|---|
| C.K. | 14.0 | |
| M.G. | 15.0 | |
| M.W. | 12.7 | |
| R.S. | 10.4 | |
| M.S.* | 17.0 | |
| D.M.* | 20.0 | 22.5 |
| Dogs | | |
| 1 | 17.1 | 15.1 |
| 2 | 16.4 | 19.8 |
| 3 | 10.6 | 14.0 |

* Subjects with chronic renal disease.

In Table III are listed the apparent volumes of distribution of radioactive ferrocyanide as well as the volume of distribution of inulin in the cases where this was determined. Only in the two subjects with chronic renal disease did the ferrocyanide "space" exceed 15% of the body weight. Both of these patients were undernourished and had slight ankle edema. Two of the normal subjects (M.W. and R.S.) had ferrocyanide "spaces" which were obviously abnormally small. In 2 of the dog experiments and in one of the patients with renal failure the inulin space actually exceeded the simultaneously measured radioactive ferrocyanide "space." These findings are in marked contrast to those of studies utilizing large (1-1.5 g) doses of non-radioactive ferrocyanide in which the ferrocyanide space always exceeded the inulin space and averaged 20% of body weight(1,2). One explanation of this discrepancy might be partial binding of the tracer dose of ferrocyanide to plasma proteins, resulting in a higher apparent concentration of ferrocyanide in plasma water than in other extracellular fluids.

To investigate this possibility *in vivo*, $Fe^{59}(CN)_6^{\equiv}$ was injected intravenously into 2 subjects with anasarca. Samples of plasma, ascitic, pleural and peripheral edema fluid were simultaneously removed at intervals of two to 36 hours after injection. The results have been expressed in Table IV as the ratio of cpm/cc of transudate water to cpm/cc of plasma water.

At equilibrium, correcting for the Donnan factor, the ratio should approximate 1.22. At no time did the ratio exceed 1.00. This held true even when the plasma concentration had fallen below a level previously attained in the transudate. This strongly suggested *in vivo* binding of radioactive ferrocyanide to plasma proteins.

*Direct measurement of binding to plasma proteins*: Dialysis and ultrafiltration technics were found unsatisfactory for evaluating the problem of protein binding. When samples of plasma containing radioactive ferrocyanide were dialyzed against isotonic saline for as long as 48 hours very little radioactiv-

ity could be detected in the saline. Similarly, when the plasma was ultrafiltered under a positive pressure of 35 cm of mercury, less than 30% of the expected radioactivity crossed the membrane. These observations did not necessarily indicate binding of ferrocyanide to protein. Surprisingly enough, aqueous solutions of radioactive ferrocyanide similarly did not freely diffuse or filter across a Visking membrane. This could not be corrected by dissolving the tracer in isotonic saline rather than water or by soaking the Visking membrane for 24 hours prior to use in non-radioactive ferrocyanide. Large amounts of residual radioactivity could be detected on the membrane after the latter had been washed for 2 to 3 minutes in running water.

As a result of these findings, electrophoretic and protein-precipitation technics were utilized. When aqueous solutions (water or saline) of radioactive ferrocyanide were electrophoresed at pH 8.6 and 7.4 no significant quantity of radioactivity was detected on the paper with the exception of the end dipping into the anodal bath. In contrast, when serum containing radioactive ferrocyanide was electrophoresed at pH 8.6 approximately 5% of the applied radioactivity was found to migrate with the albumin fraction, and at pH 7.4, approximately 12-18% of the activity migrated with this fraction. No appreciable binding to the globulin fractions was noted.

Little attempt was made in the present investigation to study quantitatively the nature of this binding. However, when $Fe^{59}$ $(CN)_6^{\equiv}$ was added to serum samples containing increasing amounts of non-radioactive ferrocyanide the fraction bound to albumin remained approximately 5% (pH 8.6) at final ferrocyanide concentrations of 1, 2, 5 and 10 mg per 100 cc. At 50 mg per 100 cc the fraction bound fell to 1%. These findings suggest that increasing absolute amounts of ferrocyanide ion are bound up to about 10 mg per 100 cc and that above this level probably little additional binding occurs.

Further evidence of protein binding was obtained by precipitating the plasma proteins with 10% trichloracetic acid. Approximately 25% of the total radioactivity in 1 cc sample of serum remained bound to the precipitated proteins after the latter had been washed 3 times in trichloracetic acid.

*Discussion.* In the present study the assumption was made that the radioactivity recovered in serum, urine and transudates represented the injected $Fe^{59}$ as ferrocyanide and that the latter was not appreciably altered in the body. The quantitative recovery of non-radioactive ferrocyanide in previous studies (1,3) suggest that this assumption is a reasonable one.

The rate of disappearance of the labelled ferrocyanide from its initial volume of distribution can be directly determined from Fig. 1. In this normal human subject the half-time value (T ½) was approximately 135 minutes. In contrast, the T½ for unlabelled ferrocyanide ion in normal dogs is approximately 40-50 minutes(1). In spite of the slower rate of disappearance in the human experiments linearity was not maintained after 160 minutes. This observation would itself constitute a strong objection to the use of the single injection technic as a method of measuring extracellular fluid volume with this tracer. One explanation for this phenomenon is that the very rapid fall in the level of ferrocyanide in the plasma causes a changing disequilibrium between the plasma and the other extracellular compartments(1). At higher concentrations, rapid renal excretion results in an increasing gradient between these compartments while at low levels the rate of transfer of Fe $(CN)_6^{\equiv}$ from interstitial fluid to plasma may exceed the rate of excretion by the kidney. When renal excretion of ferrocyanide is impaired, as in subjects with renal disease (Fig. 2), the conditions of equilibrium are approached more closely, and a slow, linear disappearance rate is maintained.

The most important reason for the more rapid rate of disappeaarnce observed in dogs as compared to humans(1) is the higher renal clearance of ferrocyanide ion in this species. Numerous studies in the dog(4,8) have demonstrated that the clearance of ferrocyanide is equal to the glomerular filtration

TABLE IV. Ratio of Concentration of $Fe^{59}(CN)_6^{=}$ in Transudate Water/Concentration in Plasma Water.

| Subject | Fluid | Time (hr) | Ratio* |
|---|---|---|---|
| M.P. | Ascitic | 2 | .22 |
| | | 8 | .63 |
| | | 24 | .90 |
| | | 36 | .90 |
| P.G. | Edema | 2 | .42 |
| | | 24 | .85 |
| | | 36 | .90 |
| P.G. | Pleural | 2 | .26 |
| | | 24 | .65 |
| | | 36 | .87 |

* Expected ratio at equilibration = 1.22.

rate. The results of the present investigation confirm the observations of Miller and Winkler(5) that the renal clearance of ferrocyanide in humans is considerably lower than the concomitant clearance of endogenous creatinine. The absolute values listed in Table I must be accepted with reservation because of the probability of significant binding of the radioactive ferrocyanide ion by the plasma proteins.

In spite of the fact that radioactive ferrocyanide did not penetrate red blood cells or enter gastrointestinal secretions, the demonstration of appreciable *in vitro* binding to plasma albumin makes it an unsuitable tracer substance for the measurement of extracellular fluid. The abnormally low volumes of distribution (Table III) and the failure of equilibration with either pleural, ascitic or edema fluid (Table IV) would support this contention. The reported similarity between the initial volume of distribution of ferrocyanide and inulin "space" or "extracellular fluid space" when large amounts of unlabelled ferrocyanide are injected is probably explained by saturation of the ferrocyanide binding capacity of plasma protein at relatively low plasma concentrations (10 mg %) of ferrocyanide. When the absolute amount of ferrocyanide injected approaches tracer quantities, however, the fraction bound to protein becomes appreciable and introduces a significant error.

The demonstration by electrophoresis that at pH 7.4, 12-18% of the added radioactivity migrated with the albumin fraction must be considered at best a rough approximation of the magnitude of the binding which might have occurred *in vivo*. The inability to use dialysis or ultrafiltration technics and the relatively low level of radioactivity in the blood at equilibration prevented adequate study of protein binding in the *in vivo* experiments.

The character of the protein-ferrocyanide bond is not clear. It does not represent combination of iron with the iron-binding-protein of the plasma, for the latter is a $\beta$ globulin (9). It may represent an example of the tendency of plasma proteins to bind a large number of inorganic anions. Human serum albumin has been shown to combine according to the law of mass action with several inorganic anions(9). This binding is to the positive charges in the protein molecule and decreases as the pH of the solution increases. A similar effect of pH was evident in the present study, since less ferrocyanide was bound at pH 8.6 (5%) than at pH 7.4 (12-18%).

*Summary.* 1) The fate and distribution of $Fe^{59}$ tagged ferrocyanide were evaluated in humans and dogs. 2) The tracer could not be detected in red blood cells, gastric juice, saliva or feces. 3) The apparent volume of distribution of the tagged ferrocyanide was frequently smaller than the assumed extracellular space, and in 3 instances was less than the simultaneously measured inulin space. 4) *In vitro* binding to plasma albumin was demonstrated by electrophoretic and protein precipitation technics. 5) Because of its binding to plasma protein and rapid excretion by the kidneys, radioactive ferrocyanide is considered unsuitable as a measure of extracellular fluid.

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## Modification of Viral Synthesis in Tissue Culture by Substituting Pyruvate For Glucose in the Medium.* (22716)

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A previous publication(1) has shown that maximal propagation of the PR-8 strain of influenza virus by *in vitro* cultures of chorioallantoic membrane was dependent on readily available sources of energy. Recent studies revealed that penetration of the virus and utilization of cellular energy for viral synthesis were dependent also on adequate concentration of $K^+$, and that pyruvate provided a more sensitive system than glucose for studying relation of potassium deficiency to virus synthesis(2). It has now been observed that, while glucose as sole carbon source for cells permits them to propagate both the parent WS and the derived neurotropic NWS strains of influenza virus, the substitution of pyruvate for glucose as carbon source permits propagation of only the parent strain WS. It appears the differences between these two strains depends not solely on host cells chosen, but also upon subtle differences in relation between viral synthesis and sources of energy for the cells.

*Materials and methods. Virus.* Influenza A viruses, strains WS(3) and NWS(4), were maintained by propagation in the allantoic sac of 10- to 11-day-old chicken embryos. All viral inocula were from allantoic fluid stored in hermetically sealed vials in a dry ice chest. Dilutions were made in phosphate buffer pH 7.3 to 7.4. Egg infectivity titrations ($EID_{50}$) and reciprocal hemagglutinin titrations (HA) were conducted by usual methods(5,6). Tissue cultures were inoculated with $10^3$ to $10^4$ $EID_{50}$. All data represent average values of 3 experiments. The 112th allantoic sac passage of strain WS was received through the courtesy of Dr. R. R. Wagner. The 114th allantoic sac passage of strain WS was used in all experiments. Strain WS showed no neurotropic properties on intracerebral inoculation of mice. Lyophilized 8th mouse brain passage of strain NWS was kindly provided by Dr. R. W. Schlesinger. The 3rd allantoic sac passage of strain NWS was used in all experiments. In 7 subsequent intracerebral passages in mice strain NWS produced a uniformly fatal disease. *Tissue cultures and substrate.* Minced and washed chorioallantoic membranes from 10- to 12-day-old chicken embryos were used in all experiments, as previously described(2). Balanced salt solution (BSS)(7) without glucose and $NaHCO_3$ was used as the basal solution. Reagent grade† carbohydrates, pyruvate, etc. were used. The final medium was adjusted to pH 7.6 with sterile NaOH before addition of tissue and virus.

*Results.* Table I summarizes data showing that chorioallantoic membrane tissue cultures (CamTC) maintained in 0.1% glucose supported multiplication of influenza virus

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