LXIII. THE DIFFUSION OF SOLUBLE IRON COMPOUNDS *IN VITRO*. THE EFFECT OF ACIDS, BASES AND ELECTROLYTES.

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Although Blaud, when in 1831 he introduced his iron pill for the treatment of chlorosis, recognised the need for administering iron in large doses, this principle was largely overlooked until recent years, when it has been revived in the treatment of hypochromic anaemia. It is recognised to-day that adequate oral iron therapy requires considerably larger quantities than were employed ten years ago.

It has always been appreciated, nevertheless, that much of the iron administered by mouth is unabsorbed and excreted in the faeces. Recently it has been shown by Heath *et al.* [1932] that in idiopathic hypochromic anaemia at low levels of haemoglobin, parenterally administered iron in the form of iron citrate is converted approximately quantitatively into haemoglobin. This observation resolves the problem of adequate oral iron therapy into a problem of the absorption of iron from the gastro-intestinal tract.

To raise the haemoglobin level of an adult by 2 % per day, which can readily be achieved in uncomplicated cases by adequate oral iron therapy, requires the absorption from the gastrointestinal tract of approximately 50 mg.[1] of metallic iron per day. The introduction of that amount of iron daily by injection of any existing iron compound intended for parenteral administration is, for the patient, always an uncomfortable and sometimes a dangerous procedure. It is obvious therefore that there is as yet no adequate substitute for the oral administration of iron. Very few disadvantages have been demonstrated in the oral administration of large doses of iron, and in every way this form of therapy is advisable in hypochromic anaemia.

A study of absorption from the gastro-intestinal tract cannot be made, of course, *in vitro*, but there are certain physicochemical relationships which may play a part in gastro-intestinal absorption which can be so studied, *e.g.* the chemical reactions which will lead to the presence of iron in a soluble form in the gastro-intestinal tract and the effect of such conditions as the hydrogen ion concentration on these reactions and on the rate of dialysis of iron.

The object of the present investigation was to determine which forms of soluble iron most readily dialyse through cellophane membranes and what conditions affect the rate of dialysis.

[1] Taking 5 litres as an average figure for adult blood volume, 15·6 g. per 100 ml. as 100 % haemoglobin and 0·34 % as the iron content of haemoglobin.

METHODS.

Complete assembly of the apparatus used for the series of dialysis experiments is shown in Fig. 1. Seamless cellophane tubing[1] was found to be the most satisfactory medium for dialysis. The tubing was cut into suitable lengths and soaked for one hour in distilled water to remove the glycerol preservative. Each section of tubing was made into a sac by double tying and sealing with

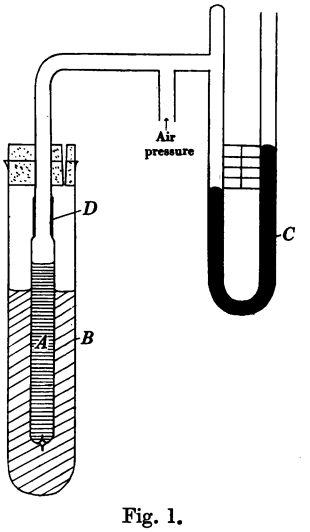

Fig. 1.

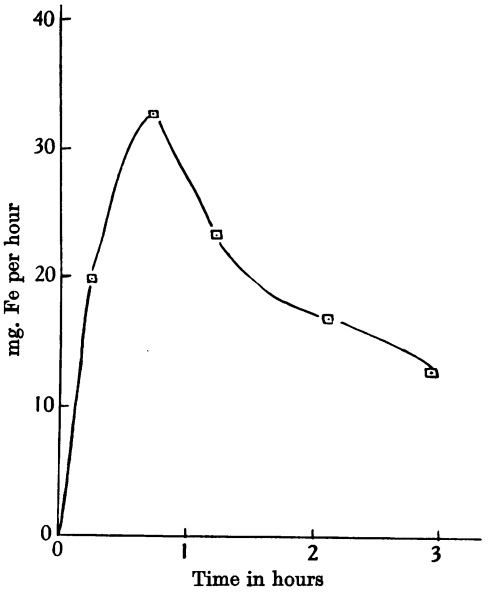

Fig. 2.

Fig. 1. Diagram of apparatus. *A*, cellophane sac containing iron mixture. *B*, diffusion medium. *C*, mercury manometer—20 mm. Hg. *D*, wax sealing cellophane on to glass tubing.

Fig. 2. Typical rate curve. Ferric ammonium citrate against serum.

collodion. Frequent checking throughout the study demonstrated that there was no leakage, and duplication of results was extraordinarily constant, varying by less than 4 % throughout the investigation. These cellophane sacs were sealed by means of paraffin wax, which made air-tight junctions, on to the ends of glass tubes projecting through rubber stoppers. The sacs were tested with applied pressure up to 40 mm. Hg. The iron solution to be studied was introduced into the sac and the diffusion medium into the outside container. Iron was subsequently determined in both the sac contents and the diffusion medium by means of a development of the Margueritte method for the volumetric determination of iron [Treadwell and Hall, 1924; Taylor and Brock, 1934]. By comparing the amount of iron recovered from the sac and from the diffusion medium at the end of the experiment with the amount introduced initially into the sac, it was possible to check accurately the entire procedure so that experimental errors were practically eliminated.

[1] Obtained from the Dupont Cellophane Company, 350, Fifth Avenue, New York, and having the following specifications: inflated diameter $\frac{3}{8}$ in., flat width 0·59 in., thickness 0·0006 in.

In order to find the optimum time for iron diffusion, the following experiment was devised. 4 ml. of a 10 % aqueous solution of ferric ammonium citrate were introduced into each of 5 sacs and diffused against normal horse-serum under an applied pressure of 20 mm. Hg. A sac was removed at the end of ½, 1, 1½, 3 and 12 hours. The rate of diffusion at each of these time intervals in mg. iron per hour was determined. These values were averaged for the time interval concerned and plotted against time. A typical curve is shown in Fig. 2. From this experiment it was observed that the curve of diffusion definitely flattens after 3 hours.

In all the experiments a constant positive pressure of 20 mm. Hg was applied to the inside of the sacs. Three different diffusion media were employed during the investigation, normal horse-serum, a special diffusion medium and distilled water. The special diffusion medium referred to in the text as "special saline," was introduced to avoid a protein precipitation which sometimes occurred with serum. It is essentially a protein-free blood-serum. The composition was based on the determination of the constituents of normal human serum as given by Mathews [1930]. 2 litres of solutions contained

Potassium sulphate	0·584 g.
Potassium chloride	0·746 ,,
Sodium chloride	11·520 ,,
Disodium hydrogen phosphate	0·564 ,,
Sodium bicarbonate	3·200 ,,
Calcium hydrogen phosphate	0·620 ,,
Magnesium chloride	0·464 ,,

The rate of diffusion was determined of a 10 % aqueous solution of ferric ammonium citrate into each of the three diffusion media.

Experimental.

First, the effect of various conditions on the rate of diffusion of ferric ammonium citrate was studied. Secondly observations were made on the rate of diffusion of some other soluble iron compounds, and the results were compared with those of ferric ammonium citrate. All experiments were performed in duplicate.

(1) *The effect of increasing concentration on the rate of diffusion of ferric ammonium citrate.*

5, 10 and 20 % aqueous solutions of ferric ammonium citrate were dialysed against normal horse-serum for the following periods: ½, 1, 1½ and 3 hours. In each case 4 ml. of the solution were introduced into the sac and 20 ml. of normal horse-serum into the container. The results are shown in Fig. 3, in which the percentage diffused was plotted against time. It will be observed that the three curves practically coincide. When these results are plotted in the form of an absolute rate curve, Fig. 4, it will be observed that the amount passing through the membrane in a given time is directly proportional to the concentration within the sac.

(2) *The effect of acid and alkali on the rate of diffusion of ferric ammonium citrate.*

10 % solutions of ferric ammonium citrate were made up in distilled water, 0·1 *N* HCl and 0·1 *N* NaOH. 4 ml. of each of these solutions were dialysed against 20 ml. of normal horse-serum for ½, 1, 1½ and 3 hours. The p_H of the

solutions varied from 1·5 in the case of the acid iron solution to 12·0 in the case of the alkaline iron solution. The results are given in Fig. 5.

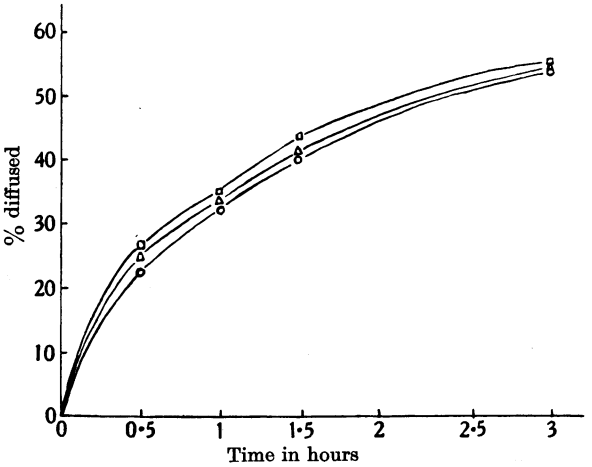

Fig. 3.

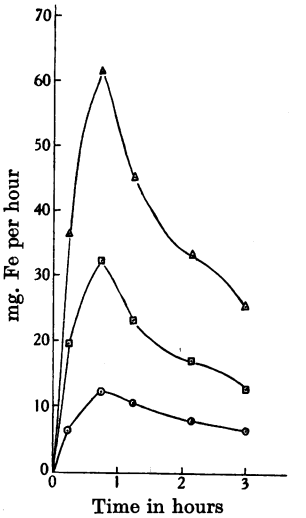

Fig. 4.

Fig. 3. Effect of concentration on the rate of diffusion of ferric ammonium citrate into serum. □ 20 %; △ 10 %; ○ 5 %.

Fig. 4. Rate curves. △ 20 % ferric ammonium citrate; □ 10 % ferric ammonium citrate; ○ 5 % ferric ammonium citrate.

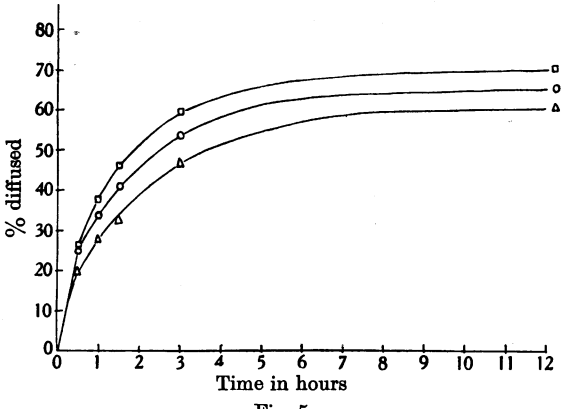

Fig. 5.

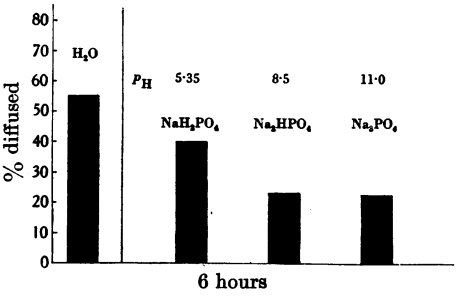

Fig. 6.

Fig. 5. Effect of acid and alkali on the diffusion rate of ferric ammonium citrate into serum. □ 0·1 *N* HCl; ○ H_2O; △ 0·1 *N* NaOH.

Fig. 6. Effect of 0·7 *M* phosphate ion on the diffusion of ferric ammonium citrate into serum.

The figure shows that the rate of diffusion from an acid medium is slightly faster than that from distilled water and the rate from an alkaline medium is slightly slower.

(3) *The effect of sodium phosphate on the rate of diffusion of ferric ammonium citrate.*

Sufficient Na_2HPO_4 was added to an aqueous solution of ferric ammonium citrate to make solutions containing 10 % ferric ammonium citrate which were respectively 0·035, 0·07, 0·35 and 0·7 *M* with respect to Na_2HPO_4. Similar solutions were made using NaH_2PO_4 and Na_3PO_4. 4 ml. of each of these 12 solutions were dialysed against 20 ml. of normal horse-serum for 6 hours. The results for the 0·7 *M* phosphate solutions are given in Fig. 6. In the case of NaH_2PO_4, the p_H of which was 5·35, there was a slight inhibition of the rate of dialysis as compared with a simple 10 % aqueous solution of the ferric ammonium citrate. In the case however of the two alkaline phosphates, the p_H values of which were 8·5 and 11·0, the inhibition of diffusion was very considerable. No precipitate whatsoever could be detected in any of these solutions. From a study of the other phosphate concentrations, it was found that the effect of the phosphate ion decreased with decreasing concentration. In the case of NaH_2PO_4, the inhibition of the rate of diffusion of the iron salt was of the same order as that found for neutral sodium chloride. For the alkaline phosphates, however, the depression of the rate of diffusion by the higher concentrations was approximately twice that of neutral sodium chloride.

The absolute rate of diffusion of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 is plotted against time in Fig. 7. Special saline was used as the diffusion medium. It will be observed that there was almost complete inhibition of the diffusion of ferric ammonium citrate and that what diffusion did occur, proceeded at a constant rate. Since no precipitate was found during the experiment, it was necessary to consider the possibility that the iron and phosphate together were modifying the capillary permeability of the cellophane membrane by forming a true adsorption compound within the pores of the membrane. Such phenomena occur when copper sulphate and potassium ferrocyanide are independently diffused through porcelain membranes, resulting in a complete change in the character of the membrane. Such adsorption compounds are usually permanently fixed in the membrane. In order to test this possibility, 10 % aqueous solutions of ferric ammonium citrate were diffused for 1·5 hours into normal horse-serum. The sacs were then washed out thoroughly in distilled water, and a 10 % solution of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 was diffused through the same membrane for 1·5 hours. The sac was once more thoroughly washed out in distilled water, and a 10 % solution of ferric ammonium citrate was diffused again through the membrane. The results are given in Fig. 8 and show that the rate of diffusion of the ferric ammonium citrate through the membrane was exactly the same after the passage of iron and phosphate together as it was before.

(4) *The effect of some other electrolytes on the diffusion of ferric ammonium citrate.*

The effect of some other electrolytes on the rate of diffusion of ferric ammonium citrate was studied. 10 % solutions of these substances—ammonium chloride, sodium citrate, sodium bicarbonate and sodium chloride—were used, in which ferric ammonium citrate had been dissolved to give also a concentration of 10 % with respect to the iron salt. 4 ml. of each of these solutions were then diffused against 20 ml. of special saline for ½, 1, 1½ and 3 hours. In this experiment special saline was preferred to serum in order to avoid the

occasional precipitation of serum-proteins by the electrolytes. The results are shown in Fig. 9, in comparison with a block for the diffusion of 10 % ferric ammonium citrate in distilled water under the same conditions. These various electrolytes decreased the rate of iron diffusion at the 3-hour period by amounts varying from 8 % for ammonium chloride and potassium citrate to 18 % for

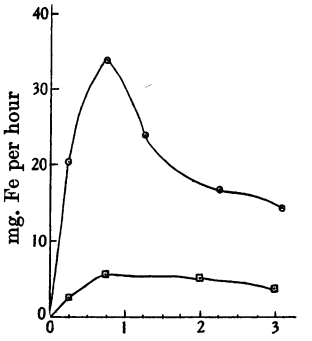

Fig. 7.

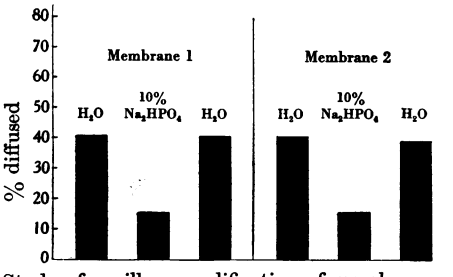

Fig. 8.

Fig. 7. Effect of phosphate. ○ Ferric ammonium citrate in H_2O; □ Ferric ammonium citrate in 0·7 *M* Na_2HPO_4.

Fig. 8. Showing the failure of ferric ammonium citrate in the presence of phosphate ion to change the characteristics of cellophane membranes.

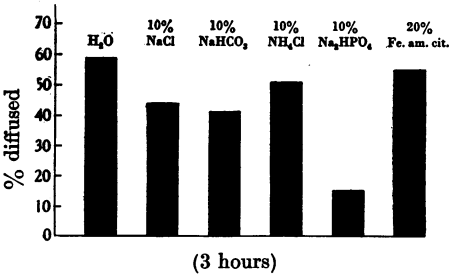

Fig. 9.

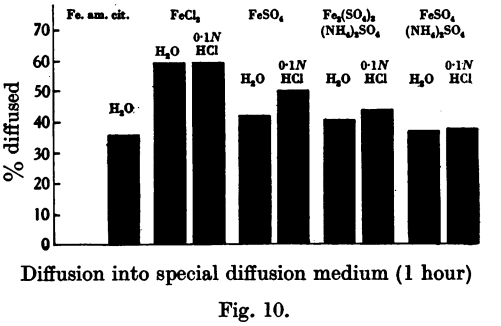

Fig. 10.

Fig. 9. Effect of various electrolytes on the diffusion of ferric ammonium citrate into special diffusion medium.

Fig. 10. Effect of acid on the diffusion of some iron salts.

sodium bicarbonate. There seems to be no relationship between the action of those electrolytes which are acid on hydrolysis and of those which react as bases. In spite of the common citrate ion, neither potassium nor sodium citrate decreased the rate of iron diffusion below that found for sodium chloride. Fig. 9 demonstrates clearly the striking inhibitory action of the alkaline phosphates on the diffusion of ferric ammonium citrate, while Fig. 6 demonstrates that the acid sodium phosphate has a much weaker inhibitory effect, which is of the same order as those of the other electrolytes tested.

(5) *A comparison between the diffusion of ferric ammonium citrate and that of some other soluble iron compounds.*

The rates of diffusion of ferric chloride, ferrous sulphate, ferrous ammonium sulphate and ferric ammonium sulphate were studied under conditions comparable with those used in the case of ferric ammonium citrate. 4 ml. of a 10 % solution of ferric ammonium citrate, which were used in that study, contained 70 mg. of metallic iron. Therefore, concentrations of the above salts were used such that 4 ml. contained 70 mg. of metallic iron. One- and three-hour periods were used in these experiments, and special saline was employed as a diffusion medium. Each of the iron salts was studied both in solution in distilled water and in 0·1 *N* HCl. The results are given in Fig. 10. The rate of diffusion of each of these salts was greater than that of ferric ammonium citrate, and with the exception of ferric chloride, the diffusion rate out of 0·1 *N* HCl was greater than out of distilled water. Ferric chloride, being an extremely acid salt, was naturally enough not affected by the presence of 0·1 *N* HCl.

All four of these soluble iron salts formed insoluble precipitates when they were added in the concentrations used in these experiments to 0·7 *M* Na_2HPO_4.

Discussion.

The data concerning the effect of increasing the concentration of ferric ammonium citrate on the diffusion of this salt need no comment. Two possible explanations can be offered for the effect of acid and alkali. The membrane itself may be modified by the presence of hydrogen or hydroxyl ions in contact with it, or the acid and alkali may interact directly with the ferric ammonium citrate to form substances having a higher or lower diffusion rate. The effect of electrolytes in general was to decrease the rate of diffusion of ferric ammonium citrate to a moderate degree. This observation is explicable on the ground that when electrolytes are present a portion of the osmotic work is being done by the electrolytes, and consequently, during any given time, a smaller number of molecules of ferric ammonium citrate pass through the membrane. Sodium dihydrogen phosphate acted in the same manner as the other electrolytes tested. The alkaline sodium phosphates however behaved quite differently. From the study of the true rate curve of iron diffusion (Fig. 7), it appears that after reaching a very low rate of diffusion the rate continues at a constant level. It was thought at first that adsorption compounds were formed within the membrane so that instead of a cellophane membrane being present, a cellophane membrane impregnated with ferric phosphate resulted. The crucial experiment described above, indicated quite clearly that such was not the case. In the absence of a precipitate the only reasonable explanation that could be offered was that the membrane became loosely plugged with a colloidal ferric phosphate. The fact that the presence of salts having a common citrate ion did not decrease the rate of diffusion below that found for sodium chloride may be considered evidence that ferric ammonium citrate diffuses through cellophane membranes largely as an undissociated molecule. From this study it has appeared that ferric ammonium citrate, which was chosen because it is a freely soluble iron compound and is used widely as a therapeutic agent, is in some ways unique. It is the only one of the five soluble iron compounds studied, which does not form a precipitate in 0·7 *M* Na_2HPO_4. Secondly, it is the only soluble iron salt which is extensively used as a therapeutic agent. The other forms of therapeutic iron in common use for oral administration being ferrum reductum and ferrous carbonate in the form of Blaud's pill.

It is of interest to compare with the above observations, certain results of investigations *in vivo* on the response of the organism to certain forms of orally administered iron. Mettier and Minot [1931] have shown that in hypochromic anaemia the reticulocyte response following oral administration of ferric ammonium citrate is greater when the iron is administered in an acid-buffered medium than when it is given in an alkaline-buffered medium. It was shown by Marlow and Taylor [1934] that the urinary excretion of iron is raised when ferric ammonium citrate is given orally in acid-buffered solutions, but not when it is given in alkaline-buffered solutions. These two observations indicate that ferric ammonium citrate, at any rate, is more readily absorbed when it is administered in an acid-buffered solution. Mettier [1930] found that the acid-buffered beef-steak medium, which he used, very materially altered the p_H of the duodenum for 2–3 hours, the time being longer when achlorhydria was present. The fact that the acid-buffered medium had a more prolonged effect in increasing the hydrogen-ion concentration of the duodenum of achlorhydric individuals than in the case of patients with a normal gastric acidity is interesting, although Mettier makes no comment on it. It appears to indicate that achlorhydric individuals are also deficient in their power to secrete alkali into the duodenum. Macallum [1893] and Abderhalden [1900] have claimed that iron is principally absorbed from the duodenum. Mitchell and Miller [1929] have shown that the ash of spinach, which contains iron, copper and manganese, is a better haemoglobin-building supplement to a milk diet in anaemic rats when the ash is dissolved in hydrochloric acid than when it is mixed in insoluble form with the food paste. It was shown by Cox *et al.* [1931] that soluble aluminium salts and ferric salts, when added to the diet of guinea-pigs in excess of the phosphorus present, cause a marked lowering of the inorganic phosphorus of the blood and of the calcium and phosphorus in the ash. This effect can be prevented by the addition to the diet of sodium dihydrogen phosphate in amount equivalent to the iron or aluminium. They state that these effects are due to the precipitation of alimentary phosphorus as the ferric and aluminium phosphates.

Brock and Diamond [1934], working with rats, have shown that the addition of large amounts of ferric or ferrous salts to a non-rachitogenic diet produces rickets. They have shown that the rickets can be prevented by the addition to this iron-containing diet of sufficient phosphate theoretically to combine with all the iron present. This work was undertaken as a sequel to the observations described in this paper on the effect of phosphates on the dialysis of iron and ammonium citrate.

So little is known about absorption from the gastro-intestinal tract that it is impossible to do more than speculate on the interpretation of some of these observations. But they do indicate the probability that reactions in the gastro-intestinal tract between iron and acids, bases and electrolytes may play an important rôle in determining the availability of that iron for absorption by the organism. Studies *in vitro* of such reactions may therefore lead to knowledge of the conditions favouring the absorption of iron.

Summary.

1. The rate of dialysis of certain soluble iron compounds across cellophane membranes has been studied under various conditions.

2. The rate of dialysis of ferric ammonium citrate is directly proportional to the concentration of the salt.

3. The rate of dialysis of ferric ammonium citrate into serum and into a special non-protein diffusion medium is increased by the presence of 0·1 *N* HCl. It is decreased slightly by 0·1 *N* NaOH, moderately by the presence of a variety of electrolytes, including sodium dihydrogen phosphate, and very markedly by secondary and tertiary sodium phosphates, in the absence of precipitates.

4. The rates of dialysis of certain other soluble iron compounds are compared with that of ferric ammonium citrate.

5. Certain observations *in vivo* recorded in the literature on the response of man and animals to various forms of iron therapy are set out for comparison.

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