

THE DEMONSTRATION OF THE IRON-BINDING GLOBULIN (TRANSFERRIN) IN SERUM AND URINE PROTEINS BY USE OF ^{59}Fe COMBINED WITH PAPER ELECTROPHORESIS

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The observation by Cartwright, Gubler, and Wintrobe (1954) that patients with the nephrotic syndrome often have a markedly reduced serum iron level associated with a lowered iron-binding capacity and an increased urinary excretion of the metal is in harmony with the theory that in this condition there is a loss of the iron-binding protein, together with other serum proteins, through the kidneys. It is now well established that the vehicle for iron transport in the body is a protein of relatively low (90,000) molecular weight (Surgenor, Koechlin, and Strong, 1949). This protein was at one time (Cohn, 1947) thought to be less specific than has since proved to be the case. It was formerly thought probable that the transport of copper, and perhaps of zinc too, was also a function of this fraction because it can be demonstrated *in vitro* that the iron-free metal-combining globulin, as it was then termed, is able to form a complex with copper.

These two metal-protein complexes, however, have different colours, the iron pink and the copper yellow. Holmberg and Laurell (1947) have described how the yellow of the copper complex was changed immediately to the pink of the iron complex on adding an iron salt to its solution. The same authors have shown that practically all of the serum copper is accounted for as the copper-containing enzyme coeruloplasmin. Their observations, including a study of the behaviour of zinc ions in competition with iron and copper for the protein, have enabled them to conclude that the sole function *in vivo* of the "metal-combining" globulin is the transfer of iron. They have given it the name "transferrin," and the preparation of its complex with iron in a crystalline state has been recently described by Laurell (1953).

The low molecular weight of this protein causes it to resemble albumin in its solubility properties,

although on electrophoresis it has the mobility of a β -globulin. It is not possible by paper electrophoresis at pH 8.6 in barbitone buffer to distinguish between this globulin and others in the β -fraction. Wallenius (1952) demonstrated that when the rat is the experimental animal the addition of radioactive iron *in vitro* to the animal's serum led to the appearance of radioactivity in the β -globulin region after electrophoresis on paper. A similar result was obtained if the iron were administered orally or parenterally and the blood withdrawn afterwards. Addition *in vitro* to human serum produced comparable results. The latter technique is employed in work reported here, the results of which have been referred to in part by Hardwicke (1954). By this simple means it has been shown that in two of the pathological conditions in which proteinuria occurs, viz., the nephrotic syndrome and congestive cardiac failure, transferrin is lost into the urine. During the course of these experiments it became clear that the reaction between iron and transferrin is very highly specific and this specificity is not impaired in the presence of a variety of serum protein abnormalities.

Since the completion of this work Horst (1954) has reported the finding of an iron binding β -globulin in ascitic fluid, pleural exudate, and in nephrotic urine.

Apparatus

The apparatus used in this study was of the hanging-strip pattern, after Durrum (1950). It was made in such a way as to avoid the disadvantage often met in this type, namely, the line of contact between filter paper and the horizontal rod or bar used to support it. By suspending the paper by the edges over glass rods bent to form semicircles it is possible completely to avoid contact of the protein bands with the support. The apparatus is illustrated in Fig. 1. Two strips of paper, Whatman No. 100 6.25 \times 45 cm.,

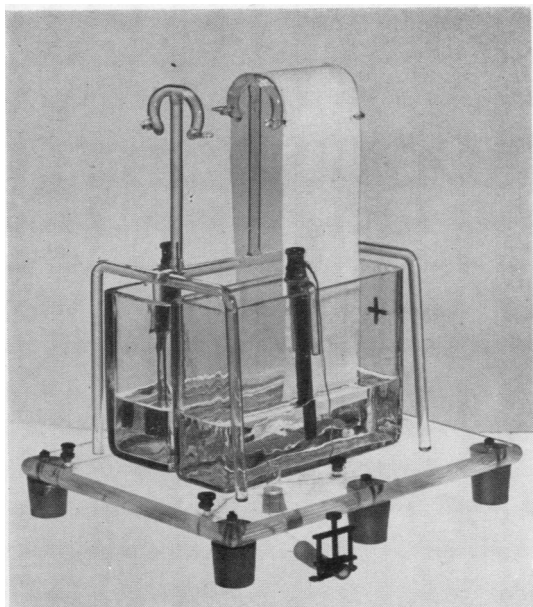


Fig. 1.—Illustration of paper electrophoresis apparatus.

can be accommodated side by side and direct current is supplied from a power pack giving a maximum of 450 volts. A large rectangular glass jar (not shown) covers the apparatus when in use. An inert gas may be admitted through the base when required.

Materials and Methods

The tracer iron used, ^{59}Fe , was in each case of a high specific activity. An early batch had an activity of $0.4 \mu\text{C}/\mu\text{g.}$, which had been improved in batches some two years later to $3.8 \mu\text{C}/\mu\text{g.}$ It was obtained as a solution of the chloride *ca.* pH 2 from the Atomic Energy Research Establishment at Harwell.

Urine and blood were collected from patients with the nephrotic syndrome and with proteinuria arising from congestive cardiac failure. To the separated serum was added ^{59}Fe to a concentration of either 0.5 or 0.05 $\mu\text{g./ml.}$ and a little solid ascorbic acid (about 5 mg./ml.). Similar additions were made to the urine, which had been first concentrated by dialysis in a collodion sac against hypertonic "dextran" solution of M.W. about 100,000. The amount of iron added was the minimum possible to obtain a satisfactory autoradiograph. By taking a larger volume of the unconcentrated urine it was found that, under the conditions used, no appreciable amount of protein was lost by diffusion through the membrane during dialysis. For the production of a urine protein separation more comparable with serum, however, it was found preferable to concentrate it first.

Of the serum-iron and urine-iron mixtures the amounts used for electrophoresis were the equiva-

lent of 0.05 ml. of serum or concentrated urine respectively. These small volumes (0.055–0.06 ml.) were spread by means of a micropipette along a pencil line drawn across the middle of a strip of filter paper, and to within approximately 1 cm. of the edges. The strip was then placed in the glass frame and buffer solution (barbitone pH 8.6, 0.05M Na barbitone, 0.01M barbitone) applied to the paper on each side just below the protein solution. It was allowed to ascend the last centimetre by capillarity. A current of 1.5 mA per strip was allowed to pass for 16 to 18 hours.

After electrophoresis the strips were dried in an oven at 100 to 105° C. and then placed in contact with Kodak "industrex D" x-ray film. Length of exposure varied from two to three weeks, after which the proteins were stained with bromphenol blue.

Results

During earlier experiments (Neale and Besford, unpublished) it had been found that both ferric and ferrous iron would enter the same position, but that uptake into the β -globulin was quicker and more complete if the iron were reduced. This is in agreement with Laurell's conclusions (1947) which were drawn from observations based on a purely chemical technique. Accordingly, a small amount of ascorbic acid, approximately 2 mg./0.5 ml., was added to the serum and urine to ensure optimal acceptance of the iron. The quantity of iron added to the serum was designed to be within the binding capacity, and a similar amount was added to the urine.

The results of electrophoresis and autoradiography are shown in Figs. 2 and 3. In each case the autoradiograph is placed to the right of its corresponding strip. Sharp black bands, the result of radioactivity, occur opposite the β -globulin bands. In Fig. 2 (b) (serum) this radioactive band is considerably sharper and more easily distinguished than the β -globulins themselves. In all four sera the added iron has been completely taken up, but three of the four urines exhibit areas behind the β -globulins which show evidence of radioactivity. This is a typical "overload" picture. Iron has been added in excess of the carrying power of the transferrin and this excess has behaved as would a solution of ferrous chloride in an oxidizing atmosphere in the absence of any protein. Under these conditions iron travels towards the anode, not the cathode (cf. Wallenius), presumably because in the slightly alkaline condition existing the hydroxide produced in the form of a sol adsorbs hydroxyl ions and becomes itself negatively charged. This charged sol is in part precipitated on the paper and in part moves in the same direc-

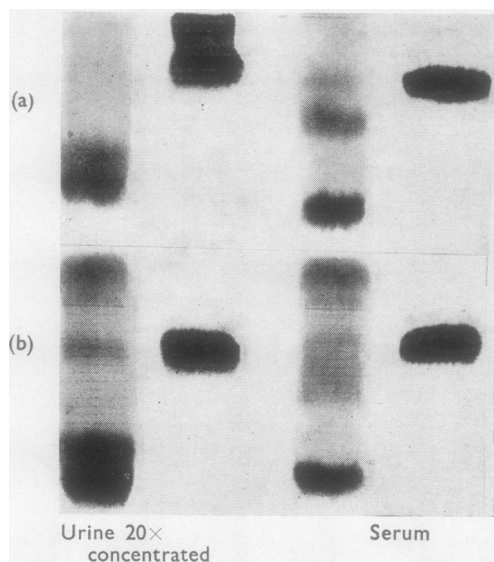


FIG. 2.—Addition of ^{59}Fe to serum and urine in two cases of the nephrotic syndrome, in these instances of $0.5\ \mu\text{g}$. (activity $0.2\ \mu\text{C}$) to each millilitre of serum or concentrated urine.

tion as the protein until oxidation occurs. A solution of ferric chloride under similar conditions is found to mordant firmly on to the paper where it is applied, although it seems to be protected somewhat by the presence of protein and can then also leave a trail. The result observed, therefore, is a combination of the tail of activity produced as described, and the sharp band corresponding in position to the transferrin.

This overloading could have occurred because of the relatively smaller amount of transferrin in the urine concentrates, and the only urine showing complete uptake (Fig. 2b) has a β -globulin band comparable in density to its corresponding serum when stained with bromphenol blue.

Although care was taken to avoid contamination of the urine, any traces of iron introduced during the collection and preparation of the specimens would be "scavenged" and held by the transferrin, partly blocking the entry of the ^{59}Fe .

Under the conditions described there is no interchange of added iron with the iron already present in complex form and where radioactivity is taken up it is indicative of the presence of unsaturated transferrin. This has been verified by adding $0.05\ \mu\text{g}$. ^{59}Fe to 1 ml. of serum withdrawn from a patient suffering from haemachromatosis whose serum iron concentration was $303\ \mu\text{g}$. per 100 ml. In this case there was only a very small proportion of the activity associated with the β -globulin band in spite of the extremely small quantity of iron

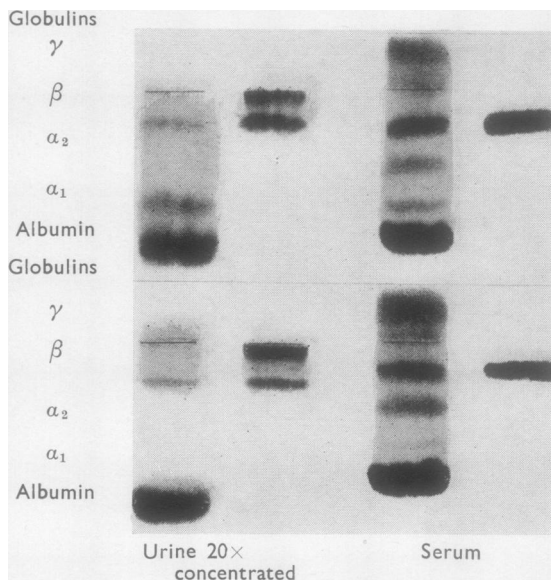


FIG. 3.—Addition of ^{59}Fe to serum and urine in two cases of congestive cardiac failure, in these instances of $0.05\ \mu\text{g}$. (activity $0.2\ \mu\text{C}$) to each millilitre of serum or concentrated urine.

added, and the autoradiograph showed almost the same picture as the control without serum.

The specificity of transferrin can be demonstrated with any serum specimen by deliberately adding iron in excess of the saturation limit, when it is seen that only transferrin accepts it, and that the excess is not, under these experimental conditions, attached to any other protein. An addition of about $2\ \mu\text{g}$. Fe per ml. is accepted by many sera before overloading, which agrees with the unsaturated binding capacity found by Laurell and other workers.

It is found that the presence of the abnormal protein found in the serum in multiple myelomatosis has no interfering effect on the iron-binding function of transferrin *in vitro*. This is true also of the large increase in α_2 -globulin which occurs in the nephrotic syndrome, and a patient with very severe hypogammaglobinaemia was found to exhibit normal iron binding. The specificity of the reaction between transferrin and iron in the presence of these several abnormal conditions argues that the appearance of an iron-linking protein having the mobility of a β -globulin in the urine of patients with proteinuria represents the leakage of transferrin through the kidneys. This finding is in accordance with modern concepts of the mechanism of proteinuria from which it would appear unlikely that any kidney should allow the escape of albumin and yet retain another protein so similar in size.

Summary

The addition *in vitro* of ^{59}Fe to the urine of patients suffering from the nephrotic syndrome and congestive cardiac failure, followed by paper electrophoresis, has been used to demonstrate the presence of transferrin, the iron-binding globulin.

A similar addition made to serum demonstrates the existence of an iron saturation limit and also the specific attachment of iron to transferrin.

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