77. Studies on Transferrin. I

The Binding of Fe^{59} with Transferrin In Vitro^{*)}

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Since Smithies' discovery,¹⁾ several different varieties of human plasma transferrin, the iron-binding β_1 globulin, have been demonstrated on the basis of their varying electrophoretic mobilities on starch gel.²⁾⁻⁹⁾ The variants are apparently genetically determined by multiple alleles designated as Tf^B , Tf^C , Tf^D , and others at a single autosomal locus.¹⁰⁾ The physicochemical properties of this protein, especially the glycoprotein nature,^{15), 16), 18)} amino acid composition, and structural mechanism of iron-binding, etc.,^{11)-14), 17)} have been studied. However, as yet, no definite correlation of phenotypic specificity with biological activity has been established.

As a preliminary step in a series of studies of the structural difference of each variant and its biological role as reflected in the ability to transport iron, this communication presents the condition of the binding of iron with one type of transferrin *in vitro*.

Experimental procedure. *Preparation of serum protein:* Immediately after taking a heparinized human blood sample by venapuncture, the sample was centrifuged at $32,700\times g$ for 10 minutes. The supernatant was subjected to starch gel electrophoresis to identify the transferrin type. Among samples isolated, type C-C was used in subsequent experiments.

Preparation of serum transferrin- Fe^{59} complex in vitro: An excess of Fe^{59}Cl solution was added to an appropriate volume of plasma, and the mixture was dialyzed in Visking Cellulose Tubing (size 24/32 or 8/32) against multiple changes of 0.001 *M* sodium phosphate buffer, pH 7.5, for 9 hours to remove excess iron ions.

Quantitative determination of transferrin- Fe^{59} complex: The amount of transferrin- Fe^{59} complex was determined quantitatively by counting Fe^{59} -radioactivity with a well-type scintillation counter (Nihon Musen Irigaku Kenkyusho). The efficiency of this apparatus was proportional to the amount of Fe^{59} , ranging from 300 c.p.m. to 30,000 c.p.m.

Chemicals: Disodium hydrophosphate was the product of Merck Co., Ltd., AG, West Germany, and all other reagents were special

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grade materials from Japanese commercial sources.

Results and discussion. Purification of the transferrin should be initiated by determining the radioactivity of Fe^{59} in the transferrin- Fe^{59} complex. For this purpose it was desirable to use a condition in which protein- Fe^{59} dissociation was minimal.

Experimental condition of removing free Fe^{59} : As shown in Fig. 1, it was possible to remove excess Fe^{59} either from the Fe^{59}Cl solution or the solution in which Fe^{59}Cl was added to serum proteins other than transferrin; about 80% of added Fe^{59} was recovered in the outer liquid after dialysis against 0.001 M phosphate buffer, pH 7.3, for 6 hours.

Fig. 1. Dialysis of free Fe^{59} in both presence and absence of serum.

- (1) Dialysis of a sample mixture of 5 ml Fe^{59}Cl and serum proteins other than transferrin (225,000 c.p.m.) against one thousand volumes of 0.05 M sodium phosphate buffer, pH 7.3.
- (2) Dialysis of a Fe^{59}Cl solution diluted with 0.001 M sodium phosphate buffer (185,000 c.p.m.) against the same buffer as (1) above.
- (3) Dialysis of sample as (1) against 0.001 M sodium phosphate buffer, pH 7.3.

Aliquots were removed from the outer liquid, and tested for radioactivity at various time intervals indicated. Outer liquid was changed four times within six hours and once after that.

In dialysis of the latter solution conducted over 20–48 hours, however, a gradual increase of radioactivity in the inner liquid was observed. This phenomenon was apparently due to denaturation of serum proteins occurring during a long-time dialysis, which resulted in precipitation of serum protein with Fe^{59} . Thus, it seemed desirable to complete the dialysis frequently changing the outer liquid within 10 hours.

Stability of transferrin- Fe^{59} binding for various pH: When the mixture was dialyzed against 0.05 M sodium phosphate buffer, the

most stable binding occurred at a pH over 7.5, and even in dialysis against buffer at pH 7.1 only 6% of the Fe^{59} was released (Fig. 2). However, in the presence of certain chelating agents, such as 0.05% EDTA, binding of iron with serum transferrin was barely stable above pH 7.8, and almost all was released below pH 7.0 (Fig. 3).

Fig. 2. The influence of pH on the stability of transferrin- Fe^{59} binding *in vitro*. Each 1 ml of Fe^{59} -serum transferrin (1,100,000 c.p.m./ml) was dialyzed against two hundred volumes of 0.05 M sodium phosphate buffer at the indicated pH's. At various time intervals 4 ml of outer liquid of dialysis were removed and tested for radioactivity.

Fig. 3. The influence of EDTA on the binding of Fe^{59} with transferrin. The conditions were essentially the same as in Fig. 1 that the buffer contains EDTA at a concentration of 0.05%.

When the transferrin- Fe^{59} complex was dialyzed against 0.05 M sodium phosphate buffer, pH 7.1 and 7.3, for 48 hours, only 6 and 4% respectively, of the original Fe^{59} was released from the complex (Figs. 4 and 5), and in dialysis against 0.01 M sodium phosphate buffer at pH 6.8 only about 2% was released. From these results it would be acceptable that the binding is more stable against buffers over pH 7.0.

Transferrin- Fe^{59} complex seemed stable, when it was dialyzed

Fig. 4. Stability of transferrin- Fe^{59} complex in dialysis against 0.05 M sodium phosphate buffer, pH 7.3. Each 0.5 ml of Fe^{59} -transferrin (980,000 c.p.m./ml) was dialyzed against two hundred volumes of 0.05 M sodium phosphate buffer. 2 ml of outer liquid and 0.02 ml of inner liquid were removed and tested for radioactivities at various time intervals.

Fig. 5. Stability of transferrin- Fe^{59} complex in dialysis against 0.05 M sodium phosphate buffer, pH 7.1. The conditions are the same as those in Fig. 4.

against 0.05 M sodium phosphate buffer at pH ranging from 7.0 to 8.0, for 48 hours either in the absence or presence of EDTA (Figs. 4–7). In these cases there was a regular inverse correlation between the radioactivities by time in the inner and outer liquids, in both of which neither turbidity nor precipitation was observed.

Influence of buffer concentration on the transferrin- Fe^{59} binding: The influence of buffer concentration on the binding of Fe^{59} with transferrin is shown in Fig. 8. It is apparent that either at pH 7.3 or 7.1 the binding is inhibited in the presence of buffer of relatively high salt concentration, that is, more than 0.05 M .

Fig. 6. Influence of prolonged dialysis on the stability of transferrin- Fe^{59} complex. Each 0.5 ml of Fe^{59} -transferrin was dialyzed against two hundred volumes of 0.05 M borate buffer containing 0.05% EDTA, pH 8.0. 2 ml of outer liquid and 0.02 ml of inner liquid were removed and tested for radioactivities at various time intervals.

Fig. 7. Stability of transferrin- Fe^{59} complex in dialysis against 0.05 M sodium phosphate buffer containing 0.05% EDTA, pH 7.0.

On checking up these results with the condition of serum proteins *in vivo*, it appears possible to form quantitatively a transferrin-Fe⁵⁹ complex without liberation of Fe⁵⁹ from the complex in the presence of buffers of concentration below 0.05 *M* and at pH ranging from 7.3 to 7.0.

Fig. 8. The influence of buffer concentration on the stability of Fe⁵⁹-transferrin binding.

— pH, 7.3
 - - - pH, 7.1

Summary. The optimal condition for the binding of Fe⁵⁹ with transferrin *in vitro* was determined.

The transferrin-Fe⁵⁹ complex was most stable in buffers of concentration below 0.05 *M* and at pH ranging from 7.3 to 7.0. In a long-time dialysis against buffers of relatively low concentration, denaturation of serum proteins occurred.

Binding was inhibited in the presence of buffers of relatively high concentration and of certain metal chelating agents such as EDTA.

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