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1 W. Z. HASSID, E. F. NEUFELD AND D. S. FEINGOLD, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 905.
2 C. A. MARSH, *Nature*, 176 (1955) 176.
3 G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 57 (1954) 275.
4 E. F. NEUFELD, D. S. FEINGOLD AND W. Z. HASSID, *J. Am. Chem. Soc.*, 80 (1958) 4430.
5 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 233 (1958) 783.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *Arch. Biochem. Biophys.*, 78 (1958) 401.
8 G. A. LEVVY, A. MCALLAN AND C. A. MARSH, *Biochem. J.*, 69 (1958) 22.
9 G. A. LEVVY, *Biochem. J.*, 52 (1952) 464.
10 D. S. FEINFOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 234 (1959) 488.
11 T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 127.
12 T. YAMAHA AND C. E. CARDINI, *Arch. Biochem. Biophys.*, 86 (1960) 133.
13 D. S. FEINGOLD, E. F. NEUFELD AND W. Z. HASSID, *J. Biol. Chem.*, 235 (1960) 910.
14 K. SHIBATA AND S. HATTORI, *Acta Phytochim. (Japan)*, 5 (1930) 117.

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The binding of Fe^{+++} to native and chemically modified human serum albumin in the presence of sodium citrate

CAMON[1] studied the binding of Fe^{+++} to bovine serum albumin over the pH range 1.5 to 3.5. His ultrafiltration experiments showed that a maximum binding of 38 atoms/mole occurred at pH 3.5 in a medium with a free-Fe^{+++} concentration of $1.7 \cdot 10^{-3}$ *M*. Ferric hydroxide precipitation at a more alkaline pH effectively limited his study. WARNER AND WEBER[2] studied Fe^{+++} binding to conalbumin in the pH range 3 to 11 and overcame precipitation by complexing the iron with citrate. They used citrate in their dialysis systems but did not find it as part of the conalbumin–iron complex. For purposes of calculation, however, they assumed a citrate–iron complex of composition 1:1 as their source of iron. BOBTELSKY AND GOLDSCHMIDT[3] studied simple complex formation between Fe^{+++} and citrate ions. Their results suggested that in the pH range 3 to 5 the complex had the composition 2 Fe^{+++}: 3 citrate ions with a net negative charge of three.

We have studied Fe^{+++} binding at near physiological pH. In view of the evidence quoted we have attempted to measure both the iron and citrate of the protein complex. Our experimental method was equilibrium dialysis at room temperature. The pH range used was 3.0 to 7.3 and the Fe^{+++} concentration was $1.8 \cdot 10^{-4}$ *M*. The buffer systems used were: pH 3.0 to 3.8, 0.05 *M* potassium hydrogen phthalate–HCl; pH 3.8 to 5.8, 0.05 *M* sodium acetate–acetic acid; pH 5.7 to 7.3, 0.01 *M* sodium cacodylate.

Each buffer was adjusted to an ionic strength of 0.16 to 0.18 with NaCl. Sodium citrate was added to give either a 1:1 or 1:5 iron: citrate ratio as required. Fe^{+++} was added as $Fe(NO_3)_3$ and tracer ^{59}Fe. All iron and citrate estimations were by isotope dilution. Protein was assayed by micro-Kjeldahl. For calculations the molecular weight of human albumin was taken as 65,500. In addition to native albumin two chemically modified albumins were studied. Acetylated albumin with 70% of the amino groups blocked was prepared by the method of MARRACK[4]. 100%-esterified albumin was prepared by a modification of FRAENKEL-CONRAT AND OLCOTT'S[5] method.

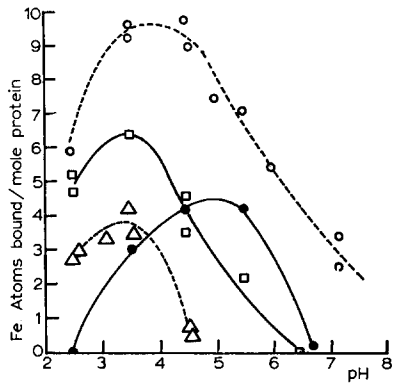

Fig. 1. ○, ●, variation of iron binding to esterified albumin; □, △, variation of native human serum albumin with pH. Solid lines represent experiments with a citrate–iron ratio 1:1. Dotted lines represent experiments using a ratio of 5:1.

The effect of pH on Fe^{+++} binding to native albumin is shown in Fig. 1. Maximum binding occurs at pH 3.9. By increasing the ratio citrate–iron from 1:1 to 5:1 the maximum iron binding is decreased. This suggests that either the albumin and citrate compete for the available iron, or that albumin combines more strongly with an iron–citrate complex of low citrate composition rather than with one with high citrate content.

We have attempted to identify the site of binding of the iron, or its citrate complex, by studying various chemically modified albumins. Acetylated albumin did not bind iron in the pH range 3.8 to 7.4. This modification has an isoelectric point which is more acid than native albumin and so our observations suggest that iron is bound to a positively charged grouping on the protein. 100%-esterified albumin showed greater binding than native albumin. This modification has an alkaline isoelectric point and would again be expected to combine predominantly with a grouping of net negative charge. Our evidence thus suggests that the iron is first complexed with citrate and that this complex, with a net negative charge, combines with the protein at some point of positive charge, such as an amino group. By decreasing the citrate–iron ratio from 5:1 to 1:1 the esterified albumin binding is considerably decreased (see Fig. 1). This suggests that the complex to be expected for binding has a citrate–iron ratio in excess of 1:1. A similar change in ratio has the opposite effect for native albumin.

We decided to measure the binding of [^{14}C]citric acid to albumin in the presence of inactive iron. At the same time an identical experiment was tried using inactive

citrate and labelled iron. The [^{14}C]citric acid was measured by the β-scintillation technique using a solid phosphor as tried by STEINBERG[6]. For native albumin the ratio of protein-bound citrate to bound Fe^{+++} is 1.5 (mean of 6 experiments). This confirms our postulate that the simple complex of BOBTELSKY AND GOLDSCHMIDT, of composition $(2Fe:3Cit)^{---}$, combines with native human serum albumin. Similar experiments with esterified albumin gave an average of 1.1 citrate ions for each Fe^{+++} bound (average of 6 experiments). We conclude that the combination with esterified albumin is in some way different from that with native albumin.

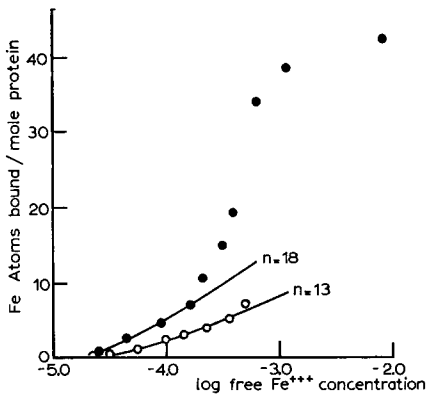

Fig. 2. O, the variation of ferric ion binding to native albumin; ●, esterified albumin with free ferric ion concentration. The solid lines represent theoretical curves for the number of binding sites indicated by n.

Experiments to assess the number of binding sites on these two proteins confirm these conclusions. Fig. 2 shows the number of atoms of iron bound/mole protein at pH 3.9 with increasing Fe^{++} concentration. For native albumin it is seen that the theoretical graph for 13 binding sites fits the experimental points. For esterified albumin, however, the theoretical binding graph for 18 similar sites does not fit the experimental points at Fe^{+++} concentrations in excess of $10^{-3.5}$ M. The interpretation of this is that sites of a different type become available beyond a Fe^{+++} concentration in excess of 10^{-4} M. We conclude that for native human serum albumin there are sites available to complex approximately 13 Fe^{+++} in the form of citrate complexes of composition $(2Fe^{+++}3Cit^{---})^{---}$. For esterified albumin there are many more sites available for a citrate-iron complex which is of uncertain composition.

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[1] L. CAMON, *Rev. acad. cienc. exact. fis. quim. y nat., Zaragoza*, 11 (1956) 35.
[2] R. C. WARNER AND I. WEBER, *J. Am. Chem. Soc.*, 75 (1953) 5094.
[3] M. BOBTELSKY AND J. M. E. GOLDSCHMIDT, *Bull. Research Council Israel, Sect. A*, 7 (1958) 121.
[4] J. R. MARRACK, *Brit. J. Exptl. Pathol.*, 35 (1954) 389.
[5] H. FRAENKEL-CONRAT AND H. S. OLCOTT, *J. Biol. Chem.*, 161 (1945) 259.
[6] D. STEINBERG, *Nature*, 183 (1959) 1253.

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