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SERUM BINDING OF VITAMIN B₁₂ ANALOGUES: IDENTIFICATION OF BINDINGGROUPS IN THE B₁₂ MOLECULE.*

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The binding of vitamin B₁₂ by serum proteins was demonstrated in exhaustive dialysis experiments¹, suggesting two binding mechanisms.^{1,2} More recent studies³ have shown that alterations in the porphyrin moiety had no effect, when compared to that of cyanocobalamin. In this paper, further studies on the sites in the porphyrin moiety for protein binding are presented. The binding capacity of serum for radio-hydroxocobalamin is compared to that for radio-cyanocobalamin, and the effect of subsequent binding of radio-cyanocobalamin by prior addition of non-radioactive analogues and sodium cyanide are presented.

MATERIALS:

Serum was obtained from 12 normal individuals, frozen and stored in the dark. Co⁶⁰-cyanocobalamin* with a specific activity of 1 $\mu\text{C}/\mu\text{g}$, and Co⁵⁷-hydroxocobalamin* with a specific activity of 0.03 $\mu\text{C}/\mu\text{g}$ were used. The hydroxocobalamin* was supplied in aqueous solution at pH 4.5 and stored frozen in the dark until used.

In figure 1 the structural formula of cyanocobalamin is shown and the sites at which substitutions may be present are indicated numerically.

Hydroxocobalamin analogue #8 has a hydroxyl group in place of the cyanide on Cobalt at position 1. Analogues 2-5 have the respective groups listed in figure 1 replacing the amino group of the propionamide of ring A, B, or C. It is not established which ring is involved, but there is

* Supplied by Dr. Charles Rosenblum of Merck & Co., Rahway, New Jersey.

** Supplied by Dr. E. Lester Smith, Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

some evidence* in favor of ring C as illustrated in figure 1.

Analogue 6 is a dibasic acid in which the amino group of 2 of the 3 underlined propionamides of ring A, B or C are replaced by OH to yield the 2 carboxylic groups; ring C is probably involved and possibly either rings A or B.

Analogue 7 is the lactam in which the encircled H's are removed and a ring formed between the acetamide chain of ring B and the attachment of the propionamide side chain on ring B.

NACN solution contained 1 millimole/ml.

METHODS:

B₁₂ binding is defined as the amount of radio B₁₂ remaining in serum after exhaustive dialysis against running tap water.¹ Radiovitamins were added at levels of 1, 2, 5, 10, 50 and 100 µg/ml of serum.

Blocking of binding sites for later binding of radio-cyanocobalamin was studied by adding a fixed amount of the cold analogue to serum (100 µg/ml serum) or 1 ml of NACN solution (1 millimole) and incubating for 2 hours at room temperature. Thereafter, the remaining binding capacity of serum for radio-cyanocobalamin was estimated. The serum pre-incubated with analogue or NACN and untreated control serum were studied in parallel to determine if blocking occurred.

*Prof. K. Bernhauer. Symposium on Vitamin B₁₂ and Intrinsic Factor, Hamburg, August 1961 (not yet published).

RESULTS:

In figures 2 and 3 radiovitamin bound after exhaustive dialysis is plotted against amount added to serum. The binding curve for cyanocobalamin consists of two phases. The steep first phase occurs when less than 5 $\mu\text{g/ml}$ of cyanocobalamin are added to serum. In the second (5 μg) smaller fractions of added cyanocobalamin remain bound after dialysis. In earlier studies¹ this two phase binding curve was interpreted as indicating two binding mechanisms (two carrier proteins and/or two sites on B_{12}). With hydroxocobalamin the binding curve shows less tendency to flatten, the binding capacity for hydroxocobalamin being greater than that for cyanocobalamin (Fig. 2). The difference is predominantly in the second phase. When analogues (nos. 2-7 Fig. 1) were added to serum prior to addition of radio-cyanocobalamin the results plotted in figure 3 were obtained. The shaded area shows the range of B_{12} binding of normal serum. The heavy line indicates the degree to which preincubation of 100 μg of unlabeled cyanocobalamin per ml of serum inhibits later binding of radioactive cyanocobalamin. Open symbols with broken line similarly indicate the effect of analogues on binding of radio-cyanocobalamin. The curves show that after substitution of the amido group of one of the propionamide side chains by the groups in analogues 2-5 (Fig. 1) these analogues retain the capacity to compete for the binding sites to the same degree as does cyanocobalamin. Filled symbols with dotted line shows that analogues nos. 6-7 (Fig. 1) inhibit binding less than does cyanocobalamin.

Hydroxocobalamin blocked cyanocobalamin binding (Fig. 4) mainly in phase II. Sodium cyanide (Fig. 5) did not block phase I significantly but blocked partially phase II binding.

DISCUSSION:

Earlier studies¹ have shown that incubation of serum with cold cyanocobalamin does not appreciably displace previously bound radio-cyanocobalamin. Under present experimental conditions, therefore, there is probably little dilution of the radiocyanocobalamin by bound cold B₁₂. The unbound cold B₁₂, however, does dilute the isotope. Such dilution may partially explain decreased isotope binding but cannot account for differences between various forms of B₁₂.

These studies give some insight into the groups on the B₁₂ molecule that are related to serum protein binding. Obviously, any interpretation of these data is based on the assumption that the structural formulae of the analogues are those given in Fig. 1.⁵ In principle, if an analogue added to serum does not block later binding of added cyanocobalamin, one can assume either that the chemical grouping which has been altered or substituted is essential for binding or that covering of this particular group alters the general reactivity of the B₁₂ molecule so that binding through some other site does not occur. In the latter case one could conclude by the use of analogues that binding occurs at a site which may in actuality not participate in the binding phenomenon. However, for our purposes we will assume that failure to block gives direct evidence of the location of the binding sites on the B₁₂ molecule. A further uncertainty lies in the fact that the precise location of the substituted side chains is not known in every case. The structure of cyanocobalamin, hydroxocobalamin and the lactam analogue is established. However, with the dibasic acid analogue the substitution is on ring C and either ring A or B (Fig. 1). With analogues 2-5 (Fig. 1) the substitution may be on ring A, B or C, but as mentioned earlier

ring C is favored.

When there is alteration of NH_2 groups on either side chain of the B pyrrol ring (lactam and dibasic acid, Fig. 1 and 2) blocking of phase I binding does not occur significantly. One can therefore assume that the CO-NH_2 groups attached to the B pyrrol ring participate in phase I binding. The analogues in which the amide side chain of the A, B or C pyrrol ring was removed or substituted significantly depressed phase I binding by subsequent addition of radiocyanocobalamin. If the location of the substitution were known with certainty, one could then state that this grouping does not participate in the binding process since the analogue was able to bind with the protein and thus prevent the later binding of radiocyanocobalamin to the protein.

The group responsible for phase II binding appears to be Co for the following reasons: The increased binding of hydroxocobalamin* indicates that either the central cobalt atom or the adjacent hydroxyl group is involved. The Co-OH bond is likely to be ionic and would dissociate in aqueous solution, leaving Co to combine with protein acceptors. Thus in the case of hydroxocobalamin, it appears that phase II binding is mediated by Co . In cyanocobalamin, the Co-CN bond is probably covalent and would have to be uncovered by removal of CN .

It is of interest to compare the mechanism of B_{12} binding by serum with that of intrinsic factor. Previous studies show that while substitution of the cyano-group in cyanocobalamin with chloro-, or thiocyanato-groups did not affect the competition for binding sites on intrinsic factor between

* Also found recently with other methods in dogs.¹²

the analogues and cyanocobalamin, the absorption of these analogues was less than that of cyanocobalamin.^{6,7,8} Modifications of the nucleotide portion of the molecule did not affect competition for binding to intrinsic factor (the side chains) but, again absorption was affected.⁸ On the other hand, replacement of benzimidazole with adenine (pseudo-vitamin B₁₂) affected both competition for binding sites and intestinal uptake.^{9,10} These studies do not indicate any correlation between intrinsic factor function and binding by intrinsic factor of vitamin B₁₂. Further studies of the relation between intrinsic factor binding of the analogues presently used are in progress. The antivitamin B₁₂ activity of various analogues has also been studied.¹¹

SUMMARY:

The binding of cyano- and hydroxocobalamin by normal serum proteins was studied. Blocking of cyanocobalamin binding by analogues with substituted amide groups in the pyrrol ring side chains was also investigated. Results suggested that some of these amide groups are involved in binding. Two phases (I and II) are seen in cyanocobalamin binding. Similarity between hydroxo- and cyanocobalamin binding in phase I, and inhibition of cyanocobalamin binding by hydroxocobalamin in phase I, suggest involvement of amide groups in phase I binding. More hydroxo than cyanocobalamin is bound, suggesting that the Co-atom is also involved in binding. The difference between hydroxo- and cyanocobalamin binding is found mainly in phase II, and the effect of NaCN on cyanocobalamin binding in the same phase also suggest involvement of Co-group in phase II binding.

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Figure 1. Structural formulae of vitamin B₁₂ and analogues used.

* IN THE DIBASIC ACID ANALOGUE THE SECOND COOH MAY BE ON THE PROPIONAMIDE CHAIN OF RING A INSTEAD OF RING B AS SHOWN. WITH EACH NUMBERED ANALOGUE ALL SIDE CHAINS NOT CARRYING THE SAME NUMBER ARE ABSENT.

FIG. 1

Figure 2. Binding capacity of hydroxocobalamin compared to cyanocobalamin in 4 normal sera.

Figure 3. Studies in competitive serum binding of "B-substituted" and "C-substituted" cobalamins. Numerals correspond to numbers of analogues in Fig. 1. Each point is the average of 4 estimations.

* FOR IDENTITY OF THE ANALOGUE SEE FIG. 1

FIG. 3

Figure 4. Study of competition of hydroxocobalamin with cyanocobalamin for binding sites in normal serum.

FIG. 4

Figure 3. Study of the effect of NaCN on serum binding capacity of cyanocobalamin.

END

FIG. 5