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From the Department of Biochemistry, College of Veterinary Medicine, Helsinki, Finland.

COBALT METABOLISM IN HORSE

SERUM LEVEL AND BIOSYNTHESIS OF VITAMIN B_{12}

By

K. Salminen

SALMINEN, K.: *Cobalt metabolism in horse. Serum level and biosynthesis of vitamin* B_{12}. Acta vet. scand. 1975, *16*, 84—94. — The levels of serum vitamin B_{12} were determined on 16 mature partly warm-blooded, partly Finnish rural-race horses by the radioisotopic competitive inhibition assay method. The mean value from three samplings carried out in dupli- or triplicate was 1.54 ± 0.16 ng/ml. The utilization of serum inorganic cobalt for cyanocobalamin synthesis was studied on two geldings, which received a dose of 200 μCi $^{58}CoCl_2$ i.v. A Sephadex G-100 gel filtration was carried out with the serum proteins from serial blood samplings at different time intervals 15 min. to 48 hrs. after administration. The gel filtration showed the presence of two labelled proteins in the serum, one of them appearing some time after administration and disappearing almost completely towards the end of the experimental period. The two elution peaks are considered to represent inorganic ^{58}Co and ^{58}Co labelled vitamin B_{12}. The appearance of labelling in serum vitamin B_{12} indicates the passing of cobalt into the intestine, and reabsorption into blood in the form of vitamin B_{12}.

cobalt; cobalt metabolism; serum vitamin B_{12}; vitamin B_{12} biosynthesis; horse; radioassay.

The importance of an adequate cobalt supply to the health of ruminants has been well documented (*Smith & Loosli* 1957, *Marston* 1970, *Smith & Marston* 1970, *Gawthorne* 1970 a). Both in vivo (*Elliot et al.* 1971, *Hedrich et al.* 1973) and in vitro (*Gawthorne* 1970 b) studies have shown that inorganic cobalt is utilized for vitamin B_{12} biosynthesis by rumen microflora, making the ruminants independent of dietary B_{12}; cobalt being the primary limiting factor for vitamin biosynthesis. Similarly some 10—50 μg of vitamin B_{12} is synthesized daily by bacteria in the human large intestine, and about the same quantity is excreted

daily in the faeces; whether any of the quantity formed by bacterial synthesis is absorbed, is doubtful (*Merzbach & Grossowicz* 1965).

Relatively little is known about the role of cobalt and B_{12} in equine nutrition. *Alexander & Davies* (1969) noted that horses consuming a natural diet had serum vitamin B_{12} levels higher than those of rat and man, equal to those of sheep and lower than those of rabbit. *Seckington et al.* (1967) carried out a quantitative study on the concentration of B_{12} in serum, and *Stillions et al.* (1971) determined the dietary requirement for B_{12} in horses.

This study was undertaken to determine the serum level of B_{12} in horses and to examine the utilization of inorganic cobalt for vitamin B_{12} biosynthesis.

The abbreviations used are: B_{12}, vitamin B_{12}; TC, transcobalamin; (^{57}Co) CN-B_{12}, (^{58}Co) CN-B_{12} and (^{60}Co) CN-B_{12}, ^{57}Co, ^{58}Co resp. ^{60}Co labelled cyanocobalamin.

MATERIALS AND METHODS

Test animals

The test animals were mature partly warm-blooded, partly Finnish rural-race horses weighing 470 to 580 kg. The horses were fed a diet composed of 6—8 kg of dry timothy alfa-alfa hay, 2½—7½ kg of barley and 100 g per day of a standard mineral-salt mixture containing cobaltous sulphate 0.015 %. For the experiment no dietary restrictions were undertaken. None had any haematological or gastroenterological problems as far as was known.

Serum vitamin B_{12} radioimmunoassay

The serum levels of B_{12} were determined by the radioisotopic competitive inhibition assay method. The endogenous vitamin B_{12} was first extracted from the binding proteins in serum, and the endogenous B_{12} admixed with exogenous ^{57}Co isotopically labelled B_{12} in the presence of a standard binder; the bound and unbound portions were then separated by centrifugation. In the standard kit* utilized, intrinsic factor was used as the binder coupled to Sephadex polysaccharide particles for the separation.

* Phadebas B_{12} Test Kit, manufactured by Ab Pharmacia, Uppsala, Sweden.

The assay procedure was carried out essentially as suggested by the manufacturer. However, due to the high B_{12} concentration in horse serum, the method was slightly modified. After the initial extraction of endogenous B_{12} from the binding proteins the assay mixture was diluted tenfold with buffer pH 4.1 containing 1.2 L-glutamic acid and 4 mg KCN in 200 ml of distilled water adjusted to pH 4.1 with 1 N-NaOH.

The ^{57}Co and ^{58}Co activity was determined by a $1\frac{3}{4}'' \times 2''$ NaJ(Tl) well-type crystal equipped with a Wallac single channel pulse height analyzer model AS-11 b. When the samples contained both ^{57}Co and ^{58}Co, the contribution of the ^{58}Co to the counts in the ^{57}Co window was eliminated by applying the appropriate correction.

Utilization of serum inorganic cobalt for cyanocobalamin synthesis

For the experiment two Finnish geldings (test horses nos. 5 and 16) were chosen, which received a dose of 200 µCi $^{58}CoCl_2$ i.v. The amount of stable cobalt as $CoCl_2$ accompanying was 18 µg. After the injection serial blood samples were taken 15 min. to 48 hrs. post administration, a total of 19 times. All sera were stored at —20°C until used. Gel filtration of the serum proteins from the $^{58}CoCl_2$ injected horses was performed through a Sephadex G-100 3 cm × 34 cm column, collecting 3 ml samples at a flow rate of 54 ml/hr. eluted by gravity with 0.1 M tris buffer pH 8.0 in 1 M-NaCl, with 0.02 % azide added as preservative, and the radioactivity of the fractions was measured. All chromatography was done at 4°C. The protein concentration was determined by measuring the absorbance at 280 nm.

RESULTS

The normal serum levels of the 16 horses studied as determined by the radioassay method, are demonstrated in Fig. 1. The lowest recorded value was 1.12 ± 0.09 ng/ml and the highest 1.74 ± 0.05 ng/ml. The mean value for all the horses was 1.54 ± 0.16 ng/ml.

The Sephadex gel filtration was carried out on serum samples of the two $^{58}CoCl_2$ injected horses. The eluate radioactivity distribution of horse no. 5 at various time intervals post administration is shown in Fig. 2. It can be seen that initially ^{58}Co became bound

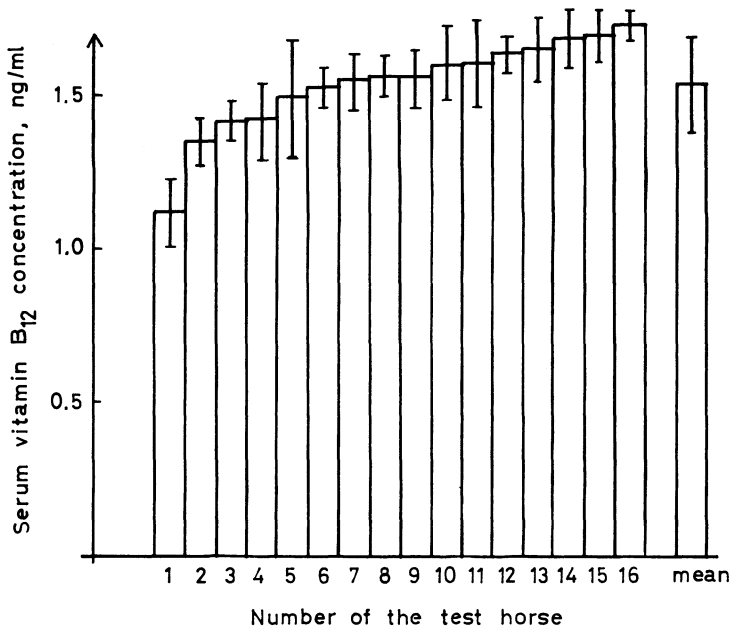

Figure 1. Serum vitamin B_{12} concentration (ng/ml) of the studied horses. Each value represents the mean ± s, derived from three determinations with three weeks' intervals in dupli- or triplicate by the radioassay method.

to a protein the molecular weight of which was somewhat higher than that of bovine serum albumin. In subsequent elutions, however, a fraction of the ^{58}Co appeared to be bound to another protein with a higher molecular weight. The elution peak of ^{58}Co bound to this protein was most prominent 4—24 hrs. post administration, and it disappeared almost completely after 48 hrs. post administration.

For the characterization of the chemical form of ^{58}Co in these protein binders, a labelling was performed. The serum samples labelled in vivo with $^{58}CoCl_2$ were mixed with serum from the same horse taken before the administration of $^{58}CoCl_2$ to which (^{57}Co) CN-B_{12} had been added in vitro, stored for 24 hrs. at 4°C and the mixture then applied to the Sephadex column. An excess of nonradioactive B_{12} was also added before the samples were mixed, to avoid possible exchange reaction involving (^{57}Co)CN-B_{12} and (^{58}Co)CN-B_{12}. When a small amount of (^{57}Co)CN-B_{12} is added to serum in vitro, it binds to the binding proteins, mainly

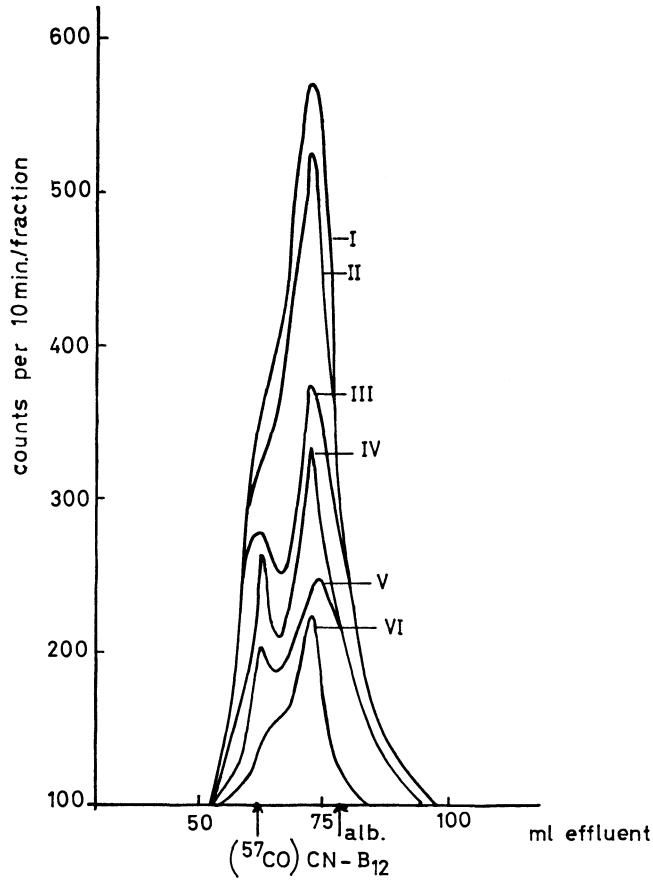

Figure 2. Sephadex G-100 gel filtration of serum from a $^{58}CoCl_2$ injected horse. The samples were taken at the following intervals post administration:
I 15 min.; II 1 hr.; III 4 hrs.; IV 12 hrs.; V 24 hrs.; VI 48 hrs.

transcobalamin II in the serum, to form a $(^{57}Co)CN\text{-}B_{12}$ TC II complex, which can then serve as an internal marker for the chromatographic experiments. Since serum from individual test animals, whose levels of TC may vary greatly, may be different, the in vitro labelling was done with serum from the same horse. The protein profiles shown in Fig. 2 reflect the chromatographic properties of the serum proteins added in vitro as well as those of the protein labelled in vivo in the test horse. Fig. 2 shows that the protein binder of $(^{57}Co)CN\text{-}B_{12}$ cochromatographs with the minor peak of ^{58}Co labelled protein binders indicating that the

biphasic elution peak observed for the protein binders of ^{58}Co labelled in vivo is due to the occurrence of ^{58}Co bound to vitamin B_{12} and as inorganic Co-ions, both forms of cobalt being bound to specific proteins. The unbound (^{57}Co)CN-B_{12} was eluted at the end of the run.

DISCUSSION

The values reported for normal B_{12} levels in horse serum seem to differ considerably. *Alexander & Davies* (1969) studied the B_{12} concentrations in the serum, urine and faeces of five horses by microbiological analysis with Lactobacillus leichmanii, and found values in the range of 6.3 ± 0.37 ng/ml to be normal. The serum levels observed by *Seckington et al.* (1967) by the same method from 25 horses as calculated from their results were 1.11 ± 0.12 ng/ml, which value is in good agreement with the results of the present study. On the other hand, the serum B_{12} level of 6.7 ± 0.42 µg/ml as reported by *Stillions et al.* (1971) is more than thousandfold higher than that observed by others, and exceeds by far the serum UBBC (unsaturated B_{12} binding capacity) of 4.6 ± 3.7 ng/ml as determined by *Rosenthal & Austin* (1962). The normal serum B_{12} levels are best known for man, and most of the recent estimates seem to vary in the range of slightly less than 200 to 800 pg/ml (*Rothenberg* 1968, *Frenkel et al.* 1970, 1973, *Wide & Killander* 1971, *Carmel* 1972a, *Raven et al.* 1972, *Kubasik & Murray* 1972); in other words, the difference between the lowest and highest values is approximately four- to fivefold. The mean B_{12} levels in serum of sheep vary in the range of 0.52 to 1.74 (*Findlay* 1972), 1.78 ± 0.58 (*Hedrich et al.* 1973) to 5.3 ng/ml (*Dawbarn et al.* 1957), and the highest value so far reported for a mammalian species is that for rabbit of 156 ng/ml (*Simnett & Spray* 1961). Even though it is known that, in general, the radioisotopic assay gives higher results than the Lactobacillus leichmanii and Euglena gracilis assays (*Raven et al.* 1969, 1972), the differences cannot be accounted for by the different methods used for B_{12} determination. It therefore seems to be normal for B_{12} levels in serum to fluctuate within a range up to fivefold, as noted for man and sheep, as well as in horse, which well explains the differences observed between the B_{12} levels of *Alexander & Davies, Seckington et al.* and the present study. The response in B_{12} production and absorption to increasing cobalt intake in the sheep described by *Elliot et al.*

(1971), *Findlay* and *Hedrich et al.* provides an explanation for the differences noted. The positive correlation between the dietary cobalt level and the B_{12} concentration in the serum as noted for sheep would seem to hold true also for the equine, so that the "normal" level of serum B_{12} varies directly within certain limits according to the cobalt content in the feed. In fact this has been experimentally proven by *Alexander & Davies,* whose results after four months' supplementing of 15 mg/day of cobaltous chloride in the feed show a distinct rise of serum B_{12} level in three test horses.

Fractionation of vitamin B_{12} enriched serum by DEAE and CM-cellulose chromatography has demonstrated the presence of two main B_{12} binders in normal human serum. Separation by electrophoresis has shown these to be an α_1 globulin and a β globulin (*Hall & Finkler* 1965, *Lawrence* 1969), which were designated as transcobalamin I and II, respectively. Sephadex gel filtration demonstrated a high molecular weight and a low molecular weight binding protein (*Hom et al.* 1966, *Hom & Olesen* 1967, *Hom & Ahluwalia* 1968). Despite the dissimilar distinctions and nomenclature, a correspondence of the first and second binders respectively of each set is often assumed. Furthermore, *Bloomfield & Scott* (1972) recently reported the identification of a third vitamin B_{12} binder (transcobalamin III) in normal human serum, and *Carmel* (1972 a, b) also reported a third vitamin B_{12} binding protein present in large amounts in a variety of conditions involving leucocytosis. Still, it should be noted that another serum B_{12} binding protein is constantly found, at least on Sephadex G-200 gel filtration, which elutes in trace amounts before the high molecular weight protein, named TC O (*Hom & Ahluwalia*). So far no reports of these vitamin B_{12} binding proteins in horse have appeared. The Sephadex G-100 gel filtration showed the presence of two labelled proteins in the serum of $^{58}CoCl_2$ injected horses, one of them appearing some hours after administration and disappearing almost completely towards the end of the 48 hrs. experimental period. Since the inorganic cobalt becomes bound to the albumin fraction (*Paley & Sussman* 1963), the two elution peaks are considered to represent the inorganic ^{58}Co and ^{58}Co labelled B_{12}. It would seem that B_{12} is bound in horse serum to a single binding protein, but due to the relative crudity of the gel filtration used, the high and low molecular weight binding proteins may be indistinguishable. However, the

cochromatography of the in vitro labelled (^{57}Co)CN-B_{12} supports the identification of the protein as a vitamin B_{12} binding protein.

The intestinal synthesis of cyanocobalamin has been known since the studies of *Dyke et al.* (1950). The site of B_{12} synthesis is considered to be the lower digestive tract, and the absorption of a small test dose of (^{60}Co)CN-B_{12} injected into the caecum was shown by the recovery of a fraction of the vitamin in urine (*Stillions et al.*). In the present study the labelled inorganic cobalt was administered intravenously, and the appearance of labelling in serum B_{12} indicated a passing of cobalt into the intestine, the site of synthesis, and reabsorption back into the blood as vitamin B_{12}. This finding is in accordance with the concept of a dynamic equilibrium between intestinal and circulating cobalt as shown earlier in chickens (*Salminen et al.* 1975). Alternatively a nonspecific exchange reaction between the inorganic cobalt pool and the vitamin B_{12} pool could be considered. However, during the in vitro labelling experiment no exchange in the opposite direction occurred, which renders the in vivo exchange reaction rather improbable. The nonsymmetric form of the elution peak as early as 15 min. after the $^{58}CoCl_2$ administration may indicate the presence of a small amount of (^{58}Co)CN-B_{12}, but the biphasic elution curve became obvious first after 2 hrs., when a sufficient amount of ^{58}Co had become incorporated into B_{12}. The labelling in serum B_{12} disappeared almost completely after two days, indicating a relatively rapid turnover of serum vitamin B_{12}.

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SAMMANFATTNING

Koboltmetabolismen hos häst. Koncentration i serum och biosyntes av vitamin B_{12}.

Koncentrationen av vitamin B_{12} i serum bestämdes hos 16 vuxna delvis varmblodiga, delvis finska hästar med hjälp av den kompetitiva radioisotop-inhibitionsmetoden. Medelvärdet av tre dubbelt eller tredubbelt undersökta prov var 1,54 ± 0,16 ng/ml. Utnyttjandet av serum oorganiskt kobolt för biosyntes av cyanokobalamin undersöktes hos två vallacker, som fick en dos av 200 μCi $^{58}CoCl_2$ intravenöst. En

Sephadex G-100 gelfiltration utfördes med serumproteiner från en serie av blodprov tagna 15 min. till 48 timmar efter injektionen. Gelfiltrationen visade, att det fanns två märkta proteiner i serum varvid den ena av dessa uppkom först en tid efter injektionen och försvann nästan helt mot slutet av försöksperioden. De två elutionstopparna ansågs representera oorganiskt ^{58}Co och med ^{58}Co märkt vitamin B_{12}. Uppkomsten av märkning serum vitamin B_{12} visar, att kobolt har passerat in i tarminnehållet, och reabsorberat i form av vitamin B_{12}.

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Reprints may be requested from: K. Salminen, State Veterinary Medical Institute, Box 368, 00101 Helsinki 10, Finland.