TRANSCOBALAMIN II-MEDIATED UPTAKE OF VITAMIN B_{12} BY RAT LIVER CELLS

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aan Inge,
Caroline en Anouk

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CONTENTS

LIST OF ABBREVIATIONS

AH-Sepharose	aminohexyl-Sepharose
BFS	vitamin B_{12}-free serum
Cbl	cobalamin
CN-Cbl	cyanocobalamin
di-CN-Cbl	dicyanocobalamin
DMEM	Dulbecco's modified minimal essential medium.
EDTA	ethylenediaminotetra-acetic acid
EGTA	ethyleneglycol-bis (2-aminoethyl) tetra-acetic acid
HBSS	Hanks' balanced salt solution
IEF	isoelectric focusing
IF	intrinsic factor
LDH	lactate dehydrogenase
OH-Cbl	hydroxocobalamin
PCA	perchloric acid
TC I, II, III	transcobalamin I, II, III
TCA	trichloroacetic acid.

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. Introduction.

Vitamin B_{12} plays a unique role in mammalian metabolism
not only because, as a coenzyme, it is involved in two
completely different and unrelated biochemical pathways, -
the synthesis of nucleic acid precursors and the catabolism
of some fatty acids -, but even more because it gives an
excellent example how different groups of living organisms
work together and depend on each other for the supply of
vital nutrients. Vitamin B_{12} is almost exclusively found in
animal products. However, it is not synthesized by the
animals themselves but, they are able in one or the other
way to absorb vitamin B_{12} which is produced by micro-
organisms. For instance in ruminants the bacteria in the
rumen are the source of the vitamin, which is taken up by
the gut, distributed over the tissues and which is
subsequently consumed by man with the meat or with the milk.
However, the quantity of vitamin B_{12}, which is available in
the food, is so low, that it would be lost if not an
elaborate system of carrier proteins and cellular receptor
mechanisms selectively collected it from the food and
delivered it to the tissues. Intrinsic factor, produced and
secreted by the gastric mucosa, binds the vitamin, which
enters the body with the food, and hands it over to the
ileal mucosa cells, which carry specific receptors for this
protein. When the vitamin enters the blood, the plasma
transport proteins, the transcobalamins, take it up
immediately and deliver it to the tissues.

It was the object of the investigations, which are
presented in this thesis, to reveal the mechanism of this

delivery and to obtain a comprehensive picture of the
various aspects of the cellular uptake. The rat liver cell
was chosen for this purpose as an in vitro model, not only
because established methods are available for the isolation
of cellular subfractions and of intact viable cells, but
also because the liver is one of the main storage pools for
vitamin B_{12} of the body and has possibly a regulatory
function in the maintenance of the blood vitamin B_{12} level.

Section 1.2. reviews the literature on vitamin B_{12} and
its binding proteins. It is, evidently, not exhaustive, but
the major steps in the progress of the understanding of
vitamin B_{12} transport and metabolism are outlined.

1.2. Literature.

1.2.1. Historical review on vitamin B_{12} investigations.
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The history of vitamin B_{12} begins with the first
description of what appears to have been pernicious anemia
by J.S. Combe in 1822 (1). In 1849 Th. Addison (2)
described a case of idiopathic anemia, which since then has
been regarded as a classic picture of severe pernicious
anemia. The name of the disease was introduced by Biermer in
1872 (3). In the years thereafter increasing evidence was
collected for a connection between the gastric and the
hematological phenomena in this disease and thanks to the
advance in morphological techniques a better description of
the megaloblastic changes in the bone marrow was obtained
by Cohnheim in 1876 (4), Ehrlich in 1880 (5), Cabot in 1903
(6) and Ehrlich in 1905 (7), and the atrophic appearance of
the gastric mucosa was revealed by Fenwick in 1870 (8) and
Cahn and von Mering in 1886 (9).

However, it lasted until 1926 before the first
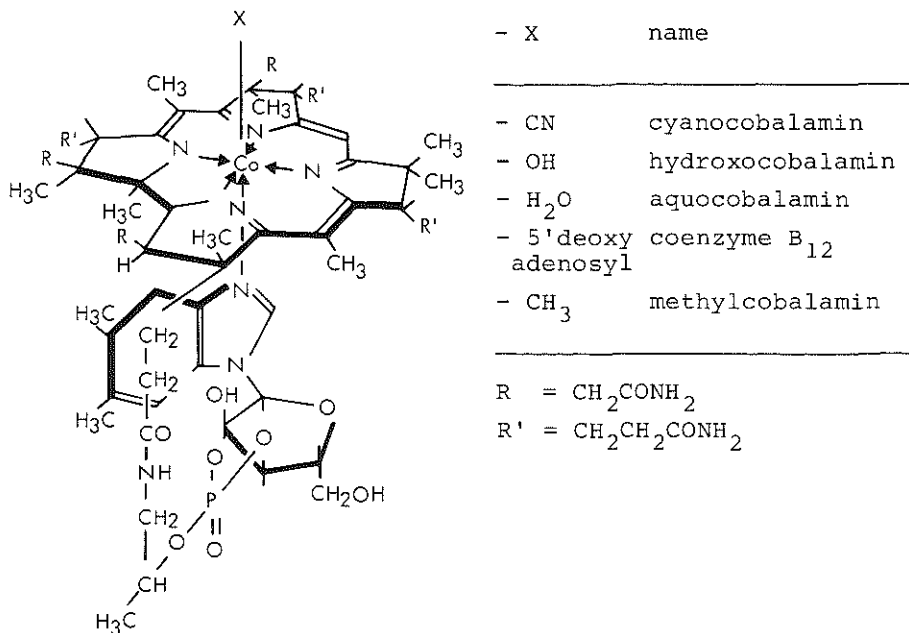therapeutic results were obtained. Using the experimental

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− X	name
− CN	cyanocobalamin
− OH	hydroxocobalamin
− H$_2$O	aquocobalamin
− 5'deoxy adenosyl	coenzyme B$_{12}$
− CH$_3$	methylcobalamin

R = CH$_2$CONH$_2$
R' = CH$_2$CH$_2$CONH$_2$

Fig. 1.1. Structural formula of vitamin B$_{12}$.

data from Whipple, Robscheit and Hooper (10), who observed
that liver was a potent hematopoietic substance in
experimentally anemic dogs, Minot and Murphy (11) treated
successfully a patient with severe pernicious anemia with
large amounts of raw liver.

The involvement of a gastric factor in the therapeutic
effect of the liver diet was first demonstrated by Castle
in 1929 (12), who reported the benificial effect of normal
human gastric juice on the therapeutic results of a beef
diet in pernicious anemia patients. In subsequent reports
Castle supposed the production of an "intrinsic factor" by
the human stomach. The "extrinsic" counterpart was assumed
to be present in the liver or beef diet.
In 1948 the extrinsic factor, named vitamin B$_{12}$, was almost
at the same time isolated in pure form by Folkers et al.
(13) in the United States and by Smith in England (14).

In 1956 Hodgkin et al. (15) revealed the structure of the vitamin B_{12} molecule by means of x-ray cristallography (figure 1.1). Finally in 1972 the last basic step in the identification of the "extrinsic factor" was set by R.B. Woodward (16), who succeeded in the complete synthesis of a biologically active vitamin B_{12}.

An important advance in the research on vitamin B_{12} transport in the body was made possible by the work of Chaiet, Rosenblum and Woodbury in 1950 (17), who incorpora- ted radioactive cobalt isotopes in the vitamin B_{12} molecule with microbiological techniques. In the first place it became possible to measure the ability of animals or patients to absorb vitamin B_{12} (18 - 21). Secondly progress was made in the purification of Castle's intrinsic factor, which is necessary for normal vitamin B_{12} absorption, by Ellenbogen et al. (22), Gräsbeck et al. (23) and Chosy and Schilling (24).

Definite physicochemical analysis could be performed after complete purification of intrinsic factor was reached first by Gräsbeck et al. in 1966 (25) using conventional techniques and later by Christensen et al. (26) and Allen et al. (27) in 1973, using affinity chromatography.

Intrinsic factor appeared to be a glycoprotein with a molecular weight of 44 000, based on aminoacid and carbo- hydrate composition. It is very selective in its binding of vitamin B_{12} molecules; cyano-, hydroxo-, methyl-, and adenosyl-cobalamin are all bound with about the same association constant of 10^{10} M^{-1}, but cobalamins with altered or lacking nucleotide portions show markedly decreased binding (27).

The availability of radioactive vitamin B_{12} made it possible to study many other aspects of the transport and metabolism of vitamin B_{12}, which will be reviewed in the following sections.

1.2.2. Absorption of vitamin B_{12}.

Vitamin B_{12} from the food is bound to intrinsic factor, which is secreted with the gastric juice, and the complex attaches to specific binding sites on the surface membrane of the epithelial cells in the distal part of the ileum (28). The demonstration of Veeger et al. (29) that patients with pancreatic insufficiency exhibit a malabsorption of vitamin B_{12} and similar observations in our laboratory with dogs suggest that pancreatic enzymes are necessary for the removal of inhibitory substances or competitive binding proteins or for the activation of intrinsic factor as suggested by Abels et al. (30). Allen et al. (31) reported that at least in man the pancreatic enzymes play a role in the destruction of non-intrinsic factor vitamin B_{12}-binding proteins in saliva and gastric juice. The vitamin B_{12}, which is almost completely bound to this type of proteins, then becomes available for intrinsic factor binding and subsequent absorption. The binding of IF-vitamin B_{12} to receptors on the brush borders of the ileal mucosa cell is the first step in the absorption process (32). This receptor was solubilized and characterized to some extent by Katz and Cooper in 1972 (33) and others (34 - 36). It is not clear what happens after binding to the receptors. The cellular uptake requires energy and it has been suggested that the total complex is taken up by the cells in a pinocytotic process (37). Some of the absorbed vitamin B_{12} is found in the mitochondria 2 hours after an oral dose of radio-active vitamin B_{12} (38). A part of the vitamin B_{12} seems to be converted to its coenzyme forms but another part reaches the blood unchanged (39). The fate of the intrinsic factor molecule is unknown. Vitamin B_{12} is released in the portal circulation with a delay of several hours and there is conflicting information on whether it is released in the free form (40) or bound to transcobalamin II (41).

1.2.3. Metabolism.

Only ten years after the first isolation of vitamin B_{12}, it was discovered that cyanocobalamin and hydroxo-cobalamin were not the active forms, which take part in cellular metabolism. The extremely low concentration of the real coenzyme forms in the tissues and their sensitivity to light, leading to rapid conversion to hydroxocobalamin, account for this. In 1958 Barker et al. (42) isolated the 5'-deoxyadenosyl-cobalamin or coenzyme B_{12}, and in 1964 Lindstrand (43) and Stahlberg (44) detected methylcobalamin by chromatographic analysis of plasma and liver extracts. Coenzyme B_{12} functions in the methylmalonyl-Co A mutase dependent reaction, in which methylmalonyl-Co A is converted to succinyl-Co A (45) and is thereby involved in the cata-bolism of branched chain and odd numbered fatty acids, some amino acids and thymine. Deficiency of the coenzyme leads to an increase in the excretion of methylmalonic acid and propionic acid in the urine.

Methylcobalamin takes part as a coenzyme in a complica-ted methylgroup transfer reaction, in which the methylgroup of methyltetrahydrofolic acid is transfered to homocysteine under the formation of methionine, whereas s-adenosyl methionine is needed as a cofactor. This reaction forms a link between sulphur-aminoacid metabolism and folate metabolism. Deficiency of the coenzyme leads to an · accumulation of methyltetrahydrofolate and homocysteine and a shortage of methionine. The accumulation of methyltetra-hydrofolate and concomittant reduction of tetrahydrofolate, the product of this reaction, causes an impairment of the synthesis of purines, and in particular of the synthesis of thymidylate from uridylate, because the folate derivatives, which are involved in these pathways are synthesized from tetrahydrofolate.

In this way vitamin B_{12} is related to the biosynthesis

of DNA precursors and a deficiency of vitamin B_{12} results, similar to a deficiency of folate, ultimately in decreased DNA synthesis and impaired cell division. The observations on the interrelationship of vitamin B_{12} and folic acid through the methyltransferase reaction were summarized in the so-called methylfolate trap hypothesis which was introduced in 1962 by Herbert and Zalusky (46) and Noronha and Silverman (47).

1.2.4. Transport of vitamin B_{12}.

After absorption of vitamin B_{12} by the epithelial cells of the ileal mucosa, the molecule is transported through the cell into the portal blood system and immediately bound to specific plasma transport proteins. The first report about plasma vitamin B_{12} binding proteins was from Pitney et al. (48) who described that endogenous vitamin B_{12} is largely present in the α-globulin fraction of plasma proteins. Miller and Sullivan (49) found that both in the α_1-globulin and β-globulin fraction binding of vitamin B_{12} took place and that in chronic granulocytic leukemia the vitamin B_{12} binding capacity of α_2-globulin fraction was highly increased.

Experiments of Hall and Finkler (50,51) with intravenously injected vitamin B_{12} showed that vitamin B_{12} bound to the beta-globulin fraction disappeared much faster from the blood than vitamin B_{12} bound to the α_1-globulin fraction. The beta-globulin binder, which they called trans-cobalamin II, to distinguish it from the α_1-globulin binder, which was called transcobalamin I, was thought to function at an earlier stage of vitamin B_{12} distribution than the α_1-globulin binder and to be important in the movement of vitamin B_{12} from intestine to tissue.

In 1969 a third binding protein in serum was described

by Lawrence (52). This binding protein has the molecular weight of transcobalamin I (TC I) and the electrophoretic mobility of transcobalamin II (TC II) and was named transcobalamin III (TC III) (53,54). In contrast to TC I the new binder exhibited the ability to transport vitamin B_{12} to the liver (55).

Different names for the vitamin B_{12} binding proteins were used. Gullberg (56) proposed transcobalamin large (TC L) for TC I and TC III and transcobalamin small (TC S) for TC II. Based on the electrophoretic mobility TC I and III were also called R-binders (rapid mobility). The R-binders are present in almost all body fluids and precise analysis of the protein from the different sources revealed that they are immunologically identical(57) and that the amino acid composition is the same for all R-binders, suggesting a common peptide backbone (58). Variation in carbohydrate composition and structure is held responsible for the microheterogeneity in isoelectric point (59,60). The distinction between TC I and TC III is therefore artificial, TC I is the acidic fraction of the R-proteins or cobalo-philins, as they were named by Stenman (60) and TC III is the more basic fraction. Cobalophilins from granulocyte lysate, which are indistinguishable from the plasma TC III fraction, have a short half life in the circulation and seem to deliver their bound vitamin B_{12} exclusively to the liver (61,62), which confirms earlier observations of Toporek et al. (55). The presence of cobalophilin in granulocytes and the tendency of these cells to release these binding proteins during serum preparation (56), have caused much confusion in the determination of the vitamin B_{12}-binding capacity of the TC III fraction in sera from normal and leukemic patients. Carmel (63) has recently lined up the differences between measurements in plasma and in serum and the effects of various anticoagulants with respect to the vitamin B_{12}-binding capacity of the three transcobalamins.

He concluded that the most reliable results are obtained with NaF/EDTA plasma and that the concentration of TC III is generally very low in normal plasma and only moderately elevated in polycythemia vera and chronic myelogenous leukemia. Highly elevated levels of TC I were found in plasma from patients with chronic myelogenous leukemia, leukocytosis and several forms of cancer.

The function of cobalophilins is still obscure, especially because no clinical symptoms were found in two patients which had a deficiency of all cobalophilins (64). Cobalophilins are not found in the rat and the mouse and their only plasma transport protein is transcobalamin II. In these animals all endogenous vitamin B_{12} is bound to this protein and there is a relatively large unsaturated vitamin B_{12}-binding capacity. Cooper and Parenchych (65) described the uptake of vitamin B_{12} by human and mouse ascites tumor cells and this uptake was completely dependent on the presence of transcobalamin II. This suggested that trans-cobalamin II is not only involved in transport of vitamin B_{12} through the blood but also takes part in the uptake mechanism of the tissues. This vital role of TC II in the delivery of vitamin B_{12} to the tissues was proven by the observations on patients with a congenital deficiency of TC II, who, despite a normal serum vitamin B_{12} concentration, developed a severe megaloblastic anemia which could only be corrected by frequent parenteral injections of large amounts of vitamin B_{12} (66 - 68).

More recent developments in the studies on the cellular uptake of vitamin B_{12} are discussed in section 1.2.6.

1.2.5. Purification and characterization of transcobalamin II.

The isolation of transcobalamin II from plasma in a

pure form is severely hampered by the very low concentration, 25 µg/l, of this vitamin B_{12}-transport protein. Rat and mouse plasma have higher concentrations but in these animals the availability of the plasma is a limiting factor.

A second problem in the purification of TC II is the unstability of the protein. At low salt concentrations the protein tends to aggregate and at a pH below 5.5 irreversible loss of vitamin B_{12}-binding capacity occurs. All these circumstances make it very difficult to purify TC II with an acceptable yield by means of conventional separation methods. Puutula and Gräsbeck (69) nevertheless reached a purification index of about 10^6 with a yield of 2.2%. Starting from 25 kg Cohn fraction III, subsequent CM-cellulose batchwise elution, DEAE-cellulose batchwise elution, recycling chromatography on sephadex G100, ammonium sulphate precipitation, DEAE-cellulose column chromatography, calcium phosphate gel chromatography, and immunoadsorption were carried out and the total product was characterized functionnally by the stimulation of the vitamin B_{12} uptake by erythrocytes and Hela cells. The Stokes radius of the isolated protein was computed at 25.5 Å , the molecular weight with gel filtration was 38 000 and with sedimentation equilibrium ultra centrifugation 28 500 and 30 100. The isoelectric points were 6.2-6.3 and 6.4-6.5; the minimum value of the 280/361 nm extinction ratio was 3.43. The protein was thought to be a glycoprotein with 13.0% neutral hexoses but no sialic acid. It was assumed that about 30% of the total protein was transcobalamin II-vitamin B_{12} complex, whereas the remainder was supposed to be partly unsaturated binder.

In 1972 Allen and Majerus published a major improvement by the use of affinity chromatography for the purification of TC II (70). A monocarboxylic derivative of cyanocobalamin was covalently coupled to 3.3'-diaminodipropylamine-substituted Sepharose using carbodiimide. 72 kg Cohn

fraction III from human plasma were dissolved and TC II was extracted with CM-Sephadex. The CM-Sephadex eluate was applied to a 10 ml cyanocobalamin-Sepharose column which was intensively rinsed with a variety of buffer systems. Finally the linkage between the immobilized ligand and the binding protein was broken by the denaturing effect of a 7.5 M guanidine solution. To remove the contaminating proteins, which were still present, the eluate was subsequently subjected to DEAE-cellulose, 3.3'-diaminodipropyl-amine-Sepharose and Sephadex G150 chromatography. The final product had a specific binding activity of 28.6 µg vitamin B_{12}/mg protein and was 2.10^6 fold purified with a yield of 12.8%. The 280/361 nm ratio was 2.04. The pure product was homogeneous in polyacrylamide gel electrophoresis, sedimentation equilibrium centrifugation, gel filtration, and the ratio of total amino acid content to bound vitamin B_{12}.

A surprising result was obtained with the determination of the molecular weight, namely 59 500 on amino acid analysis and 53 900 and 60 000 with sedimentation equilibrium centrifugation. In a later publication (71) Allen corrected this observation and confirmed the molecular weight of 38 000 found in other studies. The explanation for the higher molecular weight in the first report was probably the aggregation of a polypeptide fragment of TC II with a molecular weight of 25 000 with an intact molecule of 38 000. This fragment had probably arisen by proteolysis before or during the purification procedure. In contrast to the observations of Puutula and Gräsbeck (69), no carbohydrates were found in the pure preparation, which indicated that TC II is not a glycoprotein.

In 1975 Nexø (72) presented another affinity chromatography technique for vitamin B_{12} binding proteins which made use of the property of hydroxocobalamin to form a thermolabile linkage with 3.3'-diaminodipropylamine. The elution of the protein from the affinity column was obtained

by an elevation of the temperature, instead of the protein denaturation by guanidine as used by Allen (70). With this technique rabbit transcobalamin II was purified and characterized by Nexø et al. (73). Although no solid evidence was given for the purity of their preparation, the given analytical data are in agreement with those reported by Allen (70). Rabbit and human TC II showed to have a similar overall amino acid composition and the N-terminal sequence was found to be different from that of transcobalamin I and intrinsic factor, except for the first three amino acids which were the same for TC I and TC II.

The similarity between TC II molecules from different animal species regarding immunological and biological properties was described by Tan and Blaisdell (74). Mammalian TC II showed a high degree of mutual cross-reactivity, but avian, amphibian, reptilian and piscine TC II did not cross-react with anti-rabbit TC II. Regarding the biological activities of mammalian TC II towards reticulo-cytes or erytrocytes from different mammalian species, all preparations stimulated the binding of vitamin B_{12} to the cells but there was a great variation in the degree of promotion.

1.2.6 Cellular uptake and metabolism of transcobalamin II-vitamin B_{12}.

It was concluded from the data in section 1.2.4. that in vivo transcobalamin II plays a vital role in the transfer of vitamin B_{12} to the tissue. Also in vitro, trans-cobalamin II mediated the uptake of vitamin B_{12} by cells, for instance by reticulocyte-rich erythrocyte suspension (75) and by Hela cells (76). The uptake of transcobalamin II-vitamin B_{12} was believed to consist of a primary stage, in which binding of the complex to the cell surface takes

place, and a secondary phase, in which the transport of vitamin B_{12} into the cells occurs. An exception of the rule that TC II-bound vitamin B_{12} is taken up in higher amounts than free vitamin B_{12} seems to be the kidney. Perfusion of dog kidney with either free or TC II-bound vitamin B_{12} showed that larger amounts of free than of bound vitamin B_{12} were retained, possibly by reabsorption in the tubuli (77).

In vivo experiments with mice by Tan et al. (78) showed that with the uptake of TC II-vitamin B_{12} by the tissues the TC II is consumed. The level of TC II must therefore be maintained by de novo synthesis of the carrier protein. Heart and spleen were quite effective in the removal of TC II-vitamin B_{12} and free vitamin B_{12} from the perfusion medium in experiments of Hall and Rappazzo (79). The relatively large amount of uptake of free vitamin B_{12} was thought to be caused by production of TC II in this perfusion system (80).

The production of TC II by the rat liver was investigated by Cooksley and Tavill (81,82), who concluded that there is a continuous production of TC II, which is independent of the presence of unsaturated or saturated TC II or of the free vitamin B_{12} concentration in the perfusate. The release of TC II in the medium was inhibited by cycloheximide. Together with the production of binding protein the perfused rat liver released vitamin B_{12} molecules, taken up after in vivo injections of $\left[^{57}Co\right]$ vitamin B_{12}. The rate of release was constant and was dependent on the length of time between the injection and the perfusion. Release of radioactive vitamin B_{12} in the bile was generally low. Cycloheximide had no effect on the release, nor had extracellular TC II and free vitamin B_{12}. TC II production was in 20-fold excess of the vitamin B_{12} release on a molar base and there does not seem to be an interrelation between the release of vitamin B_{12} and the release of binding protein.

Kinetic aspects of TC II-vitamin B_{12} uptake were studied by Digirolamo and Huennekens (83). The uptake proceeded in two steps. A rapid primary step appeared to involve binding of the protein-vitamin complex to the cell membrane and had a K_m of 180 pM and a capacity of about 6 pmol/10^9 cells. This binding was hardly influenced by temperature and had an optimum at pH 5. Calcium was required for the binding but could be exchanged for magnesium. The second step was much slower and energy dependent. It was interpreted as the transfer of the vitamin to the interior of the cell. The velocity of this process was 0.4 pmol/min/ 10^9 cells. Several metabolic inhibitors were able to block this second phase. The authors concluded that these cells were able to take up vitamin B_{12} through about 400 entry sites per cell and that each entry site could transport about 10 molecules of vitamin B_{12} per hour. The final intra-cellular vitamin B_{12} concentration was about 500 times the extracellular concentration. Resuspension of the cells in TC II-vitamin B_{12}-free medium caused a rapid release of vitamin B_{12} from the cell. This observation is in agreement with the data on the release of vitamin B_{12} from the liver (80,81). The factors which are involved in the release of vitamin B_{12} from cells were investigated by Rosenberg et al. (84) in human fibroblasts. It was shown that the amount of release is at least partially dependent on the presence of an intracellular binding protein. Cultured fibroblasts from patients, who fail to synthesize both cobalamin coenzymes, defined as cbl C mutant, were unable to retain the vitamin B_{12} which was taken up during the incubation period. The intracellular protein, which bound the intracellular labeled vitamin in normal cells, was lacking in the cbl C mutant cells.

The kinetics of uptake of TC II-vitamin B_{12} by L 1210 lymphoblasts have also been studied by Ostroy and Gams (85). The incorporation of vitamin B_{12} in the cells was both

temperature and calcium dependent and was inhibited by apo-TC II (unsaturated binding protein). The K_m of the transport process was 2.5×10^{-9}M at 37°C. Again it was observed that the intracellular vitamin B_{12} could be almost completely released by the cells. The release was found to be temperature dependent: zero at 4°C and maximal at 37°C, and independent of calcium. The released vitamin B_{12} was either bound to TC II or to a protein with chromatographic properties similar to cobalophilins.

In view of their former results with the TC II dependent uptake of vitamin B_{12} by isolated rat liver mitochondria (86) Ostroy et al. expressed their doubts about the assumption that the complex, after attachment to the plasma membrane, is transfered into the cell by pinocytosis followed by lysosomal fusion and subsequent proteolysis of TC II. This model was proposed by Pletsch and Coffey (87) on the basis of studies with liver fractionation after intravenous injections of $[^{57}Co]$cyanocobalamin. At 5 min after the injection TC II-bound vitamin B_{12} was recovered in the plasma membrane fraction and at 30 min significant amounts of TC II-bound vitamin B_{12} were found in the lysosomes. At later points of time, vitamin B_{12} was mainly present in the soluble and mitochondrial compartment, but it was no longer bound to TC II. These observations suggested a dissociation of vitamin B_{12} from TC II at the level of the lysosomal system, but in the absence of a label in the protein moiety of the complex the proteolysis of TC II could not be proven.

The incorporation of a protein label became possible after the complete purification of TC II by Allen et al. (70), and the fate of pure ^{125}I-labeled TC II-$[^{57}Co]$ CN-Cbl was studied after intravenous injections into rabbits (88). The $[^{57}Co]$ CN-Cbl and the ^{125}I-label were cleared from the circulation at about the same rate and after 15-30 min ^{125}I-labeled small molecular weight-compounds were detected in

the plasma and excreted in the urine. This observation shows
that in these experimental conditions TC II is broken down
by the tissues and confirms the model as proposed by Pletsch
et al. (87). It was also noticed that a considerable part of
the vitamin B_{12} re-entered the circulation.

Further elucidation of the way of entry and intracellu-
lar transport of TC II-vitamin B_{12} was given by Youngdahl-
Turner et al. (89). Human fibroblasts were incubated with
$[^{125}I]$ TC II-$[^{57}Co]$ vitamin B_{12} under various circumstances.
^{125}I- and ^{57}Co-radioactivity were taken up by the cells
almost at the same rate. $[^{57}Co]$ vitamin B_{12} was incorporated
in the earlier mentioned large molecular weight binding
protein (84). The ^{125}I-label reappeared in the medium with
a lag of 2 hours as small molecular weight degradation
products, as a result of intracellular degradation of TC II
in the fibroblasts. The involvement of the lysosomes in this
degradation process was further made plausible by the
inhibition of proteolysis after the addition of the lyso-
somotropic agent chloroquine to the medium, which on the one
hand caused a complete inhibition of proteolysis and on the
other hand resulted in an accumulation of radioactivity
inside the cell. Probably the digestion of the complex in
the cell was blocked at the level of the lysosomes.

In conclusion there are no doubts about the crucial
role of transcobalamin II in the transfer of vitamin B_{12}
from the blood plasma to the tissues. A variety of tissues
and cell types in different animal species internalize
vitamin B_{12} from the surrounding medium much more efficiently,
when it is bound to transcobalamin II than when it is free
or bound to other vitamin B_{12}-binding proteins. Moreover it
seems to be physiologically irrelevant to study the uptake
of free vitamin B_{12} by the tissue cells, because free
vitamin B_{12} is cleared rapidly by the kidneys.

The way of entry, however, is subject to some different
views. On the one hand there is the hypothesis of pinocyto-

sis with lysosomal fusion and subsequent proteolysis of the carrier protein, which was originally based on the observations of Pletsch and Coffey (87) and further extended by the work of Allen and coworkers (88,89). On the other hand Gams and coworkers (85,86) have paid much attention to the intracellular presence of TC II and the TC II dependent uptake of vitamin B_{12} by isolated mitochondria. The latter investigators did not present a clear model for the way of entry but assumed that the intact complex enters the cytoplasma. Vitamin B_{12} can then either leak out of the cell or be taken up in the mitochondria. The fate of the carrier protein after internalization has not been investigated further.

1.3. References.

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GENERAL METHODS

2.1. <u>Materials</u>.

The following radioactively labeled compounds were
obtained from the Radiochemical Centre, Amersham, England:
cyano $[^{57}Co]$ cobalamin, code CT 2, specific activity 185-210
mCi/mg; iodine-125, code IMS 30, carrier free; $[^3H]$ inulin,
code TRA 324, specific activity 695 mCi/mmol. $[Methoxy-^3H]$
dextran was purchased from New England Nuclear, Boston, Mass.
code NET-427 B, specific activity 286.3 mCi/g. Cyano $[^{57}Co]$
cobalamin was also obtained from Philips-Duphar, Petten,
Holland, code DRN 2716, specific activity 185-210 mCi/mg.

Chromatography materials, Sephadex G 50, G 75, G 150,
G 200, Sephacryl S 200 superfine, CM-Sephadex, DEAE-
Sepharose CL-6B, CNBr-activated-Sepharose and AH-Sepharose
were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Dulbecco's modified minimal essential tissue culture
medium (DMEM) was purchased from Grand Island Biological
Company (GIBCO), Paisley, Scotland or from Flow Laboratories
Irvine, Scotland.

Cyanocobalamin (CN-Cbl) was from Merck, hydroxocobala-
min (OH-Cbl) from Sigma. All other reagents were of analy-
tical grade purity.

Male Wistar rats from the animal breeding farm of TNO
(Zeist, the Netherlands) were given food and water ad
libitum. Blood was collected under ether anesthesia by
cannulation of the abdominal aorta. The collected blood was
allowed to coagulate and the serum was obtained by centri-
fugation (10 min at 2 000 x g). The supernatant was centri-
fuged again at 100 000 x g for 1 hour and afterwards stored
frozen at $-20^{o}C$.

2.2. Measurements of vitamin B_{12} and vitamin B_{12}-binding capacity.

2.2.1. Preparation of vitamin B_{12} standard solutions.

A solution of about 0.1 mg CN-Cbl/ml H_2O was prepared and undissolved material was removed by filtration through a Büchner-funnel with filter paper S&S 589[3]. 1 ml aliquots were stored frozen at -80°C. The precise CN-Cbl concentration was determined spectrophotometrically. 4 ml CN-Cbl solution was diluted with 1 ml 0.5 M NaOH, 0.5% KCN solution, and the absorption spectrum was measured from 370 to 240 nm against 0.1 M NaOH, 0.1% KCN. At this CN^--concentration the cyanocobalamin is converted to the stable dicyanocobalamin form. The concentration was calculated using the molar extinction coefficient at the absorbance peak at 368nm: $\varepsilon \, \frac{1cm}{368} =$ 30.8 x 10^3 M^{-1}. (1).

2.2.2. Determination of vitamin B_{12}-binding capacity.

Principle: the vitamin B_{12}-binding proteins are mixed with an excess of radioactively labeled CN-Cbl. The remaining free vitamin B_{12} is removed either by coated charcoal or by gel filtration. The amount of protein-bound radioactivity is a measure for the unsaturated binding capacity of the sample.

Procedure: 0.2 ml samples from a serially diluted solution, of which the binding capacity had to be measured, were mixed with 0.9 ml 50 mM sodium phosphate, 0.15 M NaCl, 0.6 mM KCN, pH 7.4 and 0.2 ml of a CN-Cbl solution, containing 50 nCi $[^{57}Co]$ CN-Cbl per ml and CN-Cbl, adjusted to the expected binding capacity of the sample. After 30 min of incubation at room temperature the free vitamin was

separated from the bound with one of the following techniques:

1. The coated-charcoal technique. (2).

   0.3 ml coated-charcoal suspension (5% activated carbon in 0.25% dextran T 70, 0.25% bovine hemoglobin solution) was added and after centrifugation for 10 min at 2 000 x g at 4°C, the amount of ^{57}Co-radioactivity in the supernatant was measured. After subtraction of a blank value, obtained from a sample without binding protein, the vitamin B_{12}-binding capacity of the sample was calculated from the specific activity of the $[^{57}Co]$ CN-Cbl solution. The amount of charcoal is chosen in such a way that minimal protein absorption goes together with optimal free vitamin absorption. However, falsely low values due to absorption of binding protein may occur in samples with a low protein content. On the contrary the binding capacity of samples with, for instance, much lipid material may be overestimated as a result of diminished free vitamin absorption (3,4).

2. Gel filtration.

   The total incubation mixture was applied to a Sephadex G 50 column (1.6 x 35 cm) and eluted with 20 mM sodium phosphate, 1 M NaCl, buffer pH 7.4. The radioactivity in the 2 ml fractions was measured and the partition between free and bound CN-Cbl was used to calculate the Cbl-binding capacity of the sample. Gel filtration is generally accepted as reference for the separation of free and bound vitamin B_{12}. It is however much more laborious and time-consuming than the coated charcoal technique.

   One molecule of vitamin B_{12} reacts with one molecule of vitamin B_{12}-binding protein. Therefore the binding capacity, expressed as mol/l, reflects also the molar concentration of the binding proteins.
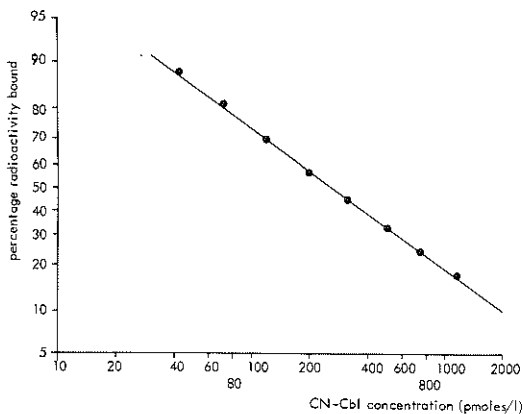
figure 2.1. A representative standard curve of the vitamin B_{12} radioassay.

2.2.3. Determination of vitamin B_{12} concentration.

Principle: the used competitive protein binding technique was first described by Lau et al. (6). After destruction of binding proteins the freed vitamin B_{12} is mixed with a fixed amount of radioactive vitamin B_{12}. A binding protein, preferentially intrinsic factor, with a binding capacity for about 80% of the labeled vitamin is added and will bind labeled and unlabeled vitamin B_{12} proportionally to their concentration in the mixture. Free and bound vitamin are separated with the coated charcoal technique. The amount of radioactivity bound in the indivi- dual samples is expressed as the percentage of the radio- activity bound in a sample without vitamin B_{12}. A standard curve is constructed with serial dilutions of a standard CN-Cbl solution. The concentration of vitamin B_{12} in unknown samples is read from this curve.

Procedure: a series of 8 dilutions from 40 to 1200 pM of the standardized CN-Cbl solution (see section 2.2.1) was prepared in "vitamin B_{12}-free serum" (BFS). The BFS was made by boiling 1 volume of human serum, diluted with 4 volumes

of 0.44 M L-glutamic acid, 0.6 mM KCN, pH 3.3 for 20 min.
The freed vitamin was removed by passage over a Sepharose
column, to which an excess of intrinsic factor (IF) was
covalently coupled. All vitamin B_{12} was retained by the
immobilized IF. The eluate is a perfect medium for the
standard CN-Cbl samples because their composition becomes
identical with the composition of unknown serum samples.
When the vitamin B_{12} concentration has to be measured in
samples with a very low protein content, for instance
purified TC II preparations, the most reliable results are
obtained when these samples are diluted with BFS.

0.2 ml of a standard solution or an unknown sample was
diluted with 0.8 ml BFS or 0.8 ml 0.44 M L-glutamic acid,
0.6 mM KCN, pH 3.3, boiled for 15 min and mixed thoroughly
with 0.2 ml $[^{57}Co]$ CN-Cbl ($\pm$ 45 pg). After the addition of
0.2 ml IF solution the tubes were incubated for 45 min at
room temperature in the dark. Hereafter 0.3 ml Hb-dextran
coated charcoal suspension (see 2.2.2) was added and after
10 min the sample was centrifuged for 10 min at 2 000 x g.
The radioactivity was counted in 1 ml of the supernatant.
The blank value (a sample without binding protein) was
subtracted, the duplicate results were averaged and
expressed as the percentage of radioactivity bound (p) in
comparison with the 100% value of the zero CN-Cbl standard,
A standard curve was composed from the known CN-Cbl samples,
in which the Ln $\frac{p}{100-p}$(logit) was plotted against the log
concentration CN-Cbl.

The concentrations of vitamin B_{12} in the unknown samples
were read from the standard curve or calculated with a
programmable calculator. A representative standard curve is
presented in figure 2.1. The interassay reproducibility is
indicated by the coefficient of variation in 8 different
independent determinations: 8% at 100 pM, 7% at 210 pM and
4.4% at 630 pM.

2.3. Partial purification of transcobalamin II.

2.3.1. Transcobalamin II from rat plasma.

Normal rat serum contains 555 ± 120 pM endogenous vitamin B_{12} (mean $\pm$ S.D., n=9) and the unsaturated CN-Cbl-binding capacity ranges from 1.7 to 2.5 nmoles/l. TC II is the only vitamin B_{12}-binding protein in rat plasma and all endogenous vitamin B_{12} is bound to this protein. Partially purified rat TC II therefore always contains 15 to 20% of endogenous Cbl. For studies of binding and uptake of TC II-Cbl it is desirable to remove the greater part of the other plasma proteins, because these proteins may interfere in the experiments. Sephadex G 150 offers a good separation of TC II from most of the other plasma proteins (figure 2.2). In this particular separation 8 ml of rat serum was saturated with 3.5 µCi $\left[{}^{57}Co \right]$ CN-Cbl ($\pm$ 12 pmoles) and applied to the Sephadex column. Elution was carried out with 20 mM Tris, 1 M NaCl, pH 7.4. The high salt concentration is necessary to avoid complexing of TC II and adsorption onto the chromatography material. The TC II-containing fractions are pooled, concentrated by ultrafiltration and dialyzed against the buffer solution, in which TC II has to be used in the respective binding or uptake studies. Further purification by for instance ion-exchange chromatography leads to considerable loss of binding activity and only little improvement in purity. The final product is about 40-fold purified.

2.3.2. Transcobalamin II from human plasma.

Human plasma contains only 0.5 to 1.0 nmole TC II per liter and is therefore not an ideal starting material for a purification. However, 30 to 40% of TC II is recovered in
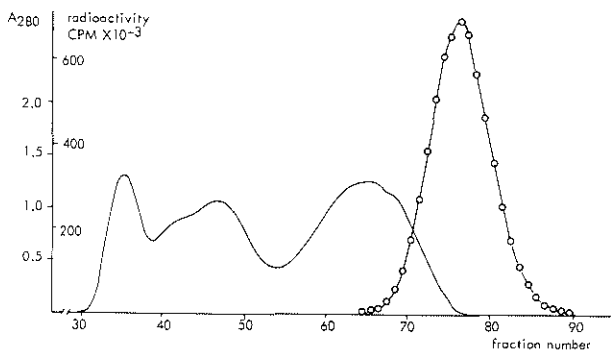
figure 2.2. Separation of 8 ml normal rat serum, satured with 3.5 μCi $\left[^{57}Co\right]$ CN-Cbl on Sephadex G 150. Elution buffer: 20 mM Tris, 1 M NaCl, pH 7.4. —————Extinction at 280 nm; o—————o, ^{57}Co-radioactivity.

Cohn fraction III from human plasma. The large scale purification of TC II from human Cohn fraction III is described in chapter 5. The affinity chromatography step in this procedure makes it impossible to incorporate a radioactive label with high specific activity in the vitamin moiety. An intermediate product from this purification, the CM-Sephadex eluate was prepared as follows: 20 kg Cohn fraction III was dissolved in 80 l of 10 mM sodium phosphate 100 mM NaCl pH 5.2. 100 g CM-Sephadex was added and after 2 hours of continuous stirring the Sephadex was collected by filtration through nylon gauze. The gel was washed with 10 l of 10 mM sodium phosphate, 50 mM NaCl, pH 5.2 and finally TC II was eluted with 1500 ml of 0.2 M Tris, 1 M NaCl pH 8.25. The greater part of the eluted material was further processed for the complete purification of TC II but small aliquots were kept apart for the partial purification of TC II-$\left[^{57}Co\right]$ CN-Cbl.
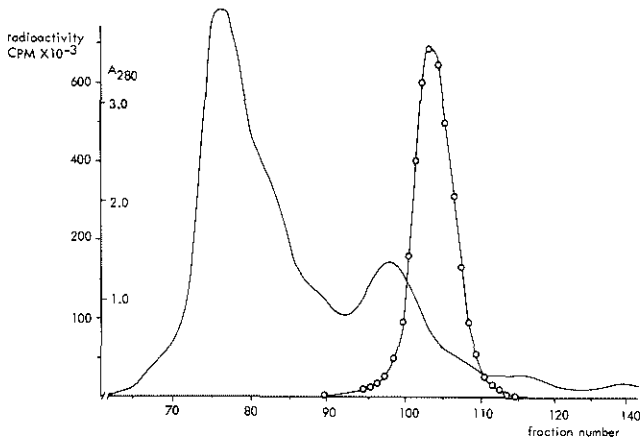
2 ml CM-Sephadex eluate (binding capacity 46.5 ng CN-

Figure 2.3. Separation of 2 ml CM-Sephadex eluate from human Cohn fraction III, saturated with 18 μCi $[^{57}Co]$ CN-Cbl, on Sephacryl S 200 Superfine. Elution buffer: 20 mM Sodium phosphate, 1 M NaCl, 0.02% NaN$_3$, pH 7.4. ————,Extinction at 280 nm; o————o,^{57}Co-radioactivity.

Cbl/ml and 0.57 ng CN-Cbl/mg protein) was saturated with 18 μCi (18 ml) $[^{57}Co]$ CN-Cbl (188 μCi/μg). After 15 min at room temperature the solution was concentrated by ultra-filtration to about 4 ml and centrifuged for 30 min at 50 000 x g. The supernatant was applied to a Sephacryl S 200 superfine column (2.6 x 90 cm). Elution took place with 20 mM sodium phosphate, 1 M NaCl, 0.02% NaN$_3$, pH 7.4. The elution profile is given in figure 2.3. The TC II-containing fractions were pooled and concentrated by ultrafiltration and meanwhile the buffer was changed to 50 mM Tris-HCl, pH 8.25. The concentrate was centrifuged at 50 000 x g for 30 min and applied to a DEAE-Sepharose CL-6B column (3 ml bed volume), which was equilibrated with the same Tris buffer. Elution took place with a concave NaCl-gradient, which was created by a two-pump system according to Lakshmanan et al. (7): a 790 mM NaCl solution in 50 mM Tris pH 8.25 is pumped into a mixing vessel with 200 ml Tris
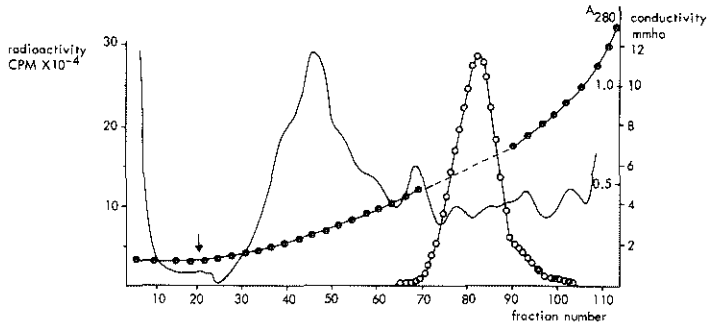
figure 2.4. Separation of the TC II-containing fractions of
          the Sephacryl column on DEAE-sepharose CL-6B.
          Starting buffer: 50 mM Tris-HCl, pH 8.25. The
          arrow indicates the start of the NaCl gradient,
          which ends at about 150 mM NaCl in 50 mM Tris-
          HCl, pH 8.25. ————,Extinction at 280 nm;
          o————o,^{57}Co-radioactivity; •————•,conductivity
          of the elution buffer.

buffer at a rate of 1.18 ml per hour. The pump rate from the
mixing vessel to the column was 15.8 ml per hour. The form
of the salt gradient is presented in figure 2.4 together
with the protein and radioactivity profile. The $\left[^{57}\text{Co}\right]$ CN-
Cbl-containing fractions were pooled and concentrated by
ultrafiltration and dialyzed against tissue culture medium
(DMEM) for later incubations with isolated liver parenchymal
cells. The specific binding activity of the purified TC II
was 42.7 ng CN-Cbl/mg protein.

2.4. Preparation of isolated rat liver parenchymal cells.

    Rat liver parenchymal cells were isolated by a colla-
genase perfusion procedure (8) according to Seglen (9,10).

Normal male Wistar rats (200-300 g) were anesthetized with
diethylether and the abdomen was opened. The portal vein was
cannulated with a Braunule 0.5G18 cannula and the liver was
perfused in situ with approximately 400 ml calcium-free
bicarbonate buffered Hanks' balanced salt solution (HBSS) at
$37^{O}C$ at a flow rate of 25 ml/min. The inferior vena cava was
cut below the liver to allow efflux of the perfusate. A
mixture of 5% CO_2 and 95% O_2 bubbled through the perfusate
to maintain a pH of 7.4 and optimal oxygen saturation. After
10 min the perfusate was changed to HBSS with calcium and
0.1% collagenase (Sigma Type II). By means of a Braunule
1G16 cannula through the right atrium into the inferior vena
cava and after ligation of the vena cava just below the liver
the perfusate could be recollected and recirculated. After
15 min the perfusion was ended and the liver, which was
soft and leaky, was excised. By means of a sharp razor blade
the liver was minced and incubated for another 15 min in
fresh collagenase medium in a mildly shaking water bath. The
cell suspension was filtered over a 63 µ nylon gauze and
washed with 100 ml of icecold HBSS. The filtrate was
collected on melting ice. The cell suspension was
centrifuged at 50 x g for 4 min. The cell pellet was gently
resuspended in 100 ml HBSS and centrifuged again. This
procedure was repeated twice with another 100 ml of HBSS and
once with tissue culture medium(DMEM) containing 0.09% human
serum albumin and buffered with 25 mM $NaHCO_3$ at pH 7.4. The
cells were finally resuspended in DMEM to a concentration
of 3.6 x 10^6 cells/ml.

This procedure consistently resulted in a cell yield
of (450 $\pm$ 100) x 10^6 cells (mean $\pm$ S.D., n=20) per liver.
The viability of the isolated cells, tested by the ability
of the cells to exclude trypan blue (0.2% in saline) was
usually better than 95%. The integrity of the cells during
incubations was followed by measurement of the leakage of
the cytoplasmic enzyme lactate dehydrogenase (LDH) into the

medium. Before and during the incubations the enzyme
activity in the cell free medium was determined according
to Bergmeyer (11) and expressed as the percentage of activity
with respect to the enzyme activity in a sonicated cell
suspension. During the incubation a gradual increase in LDH
activity in the medium was observed from 2.8% at zero time
to 7.3% at 120 min (mean of 3 experiments).

2.5. Silicon oil filtration/centrifugation technique.

For the separation of the liver cells from the medium
as a termination of the process of binding and uptake,
centrifugation through silicon oil was used (12). In this
system 0.5 ml samples were taken from the cell suspension
and layered on 0.6 ml silicon oil (Tegiloxan AV 100,
Goldschmidt, Germany), which rested on 0.1 ml 10% perchloric
acid, in a 1.5 ml micro test tube. The test tubes were
centrifuged in an Eppendorff microcentrifuge for 1 min at
8 000 x g. Aliquots from the cell-free supernatant were kept
for later analysis, most of the oil was removed by suction
and the PCA layer with the cell pellet was measured for
radioactivity after cutting the tubes just above the
interface between the PCA layer and the oil.
This method has the advantage that the cells are
completely separated from the medium and that there is no
danger for removal of surface-bound components as with
normal centrifugation procedures, which have to be followed
by several washing steps. Unfortunately, the cells take
with them a thin film of medium through the silicon oil. The
amount of radioactivity in this film has to be subtracted
from the total amount of radioactivity in the cell pellet
in order to obtain the correct amount of bound and intra-
cellular radioactivity. An estimate of the amount of medium
which is sedimentated with the cells was obtained by the

addition of a radioactive marker which is not bound and/or
taken up by the cells. The distribution of radioactivity
between the PCA layer and the medium after centrifugation
was taken as a measure for the extracellular medium space.
$[^3\text{H}]$ inulin and $[^3\text{H-methoxy}]$ dextran were chosen as inert
markers. $[^3\text{H}]$ inulin was used in a concentration of 0.4 mM
(1.1 µCi/ml). The cell suspension in DMEM with 0.09% human
serum albumin (Behringwerke, Germany) contained 2×10^6 cells
per ml. After centrifugation 50 µl aliquots from the PCA
layer and 100 µl aliquots from the cell free medium were
counted for radioactivity in triton X100 - toluene
scintillation cocktail. Duplicate samples were taken from
two different cell suspensions at 5, 30, 60 and 120 min and
the mean percentage of extracellular medium was determined
to be (1.07 ± 0.05) % (mean $\pm$ S.D.) of the 0.5 ml sample.
There was no significant difference between the two cell
suspensions or between the samples after different times
of incubation. When the sample experiment was carried out
with 25 nM $[^3\text{H-methoxy}]$ dextran (molecular weight 70 000),
the mean percentage of extracellular medium was (0.97 ± 0.05)
% (mean $\pm$ S.D.).

An even lower value was obtained when liver cells were
incubated with the plasma cobalamin transport protein
"cobalophilin", in particular the fraction with low iso-
electric point, also known as transcobalamin I. Vitamin B_{12},
bound to this protein, is not bound or taken up by the cells
(see 1.2.4). Cobalophilin-$[^{57}\text{Co}]$ CN-Cbl was added in a
concentration of 500 pM and the amount of ^{57}Co-radioactivity
in the PCA layer was found to be (0.75 ± 0.12) % of the
total radioactivity in the sample (mean $\pm$ S.D., n=13).

With these extracellular markers, three slightly
different values for the extracellular space in the PCA
layer are found. Although the experiments with the cobalo-
philin possibly give the best imitation of the incubations
with TC II, the better reproducibility of the methoxydextran

experiments has made us choose the value obtained with this
marker.

The correct values for binding and uptake of TC II-
vitamin B_{12} by the liver cells in suspension are obtained
by subtraction of 0.97% of the radioactivity in the 0.5 ml
samples from the total radioactivity in the cell pellet. All
data for binding and uptake by liver cells in the chapters
4 and 6 have been corrected this way.

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BINDING SITES FOR TRANSCOBALAMIN II-VITAMIN B_{12} ON ISOLATED RAT LIVER PLASMA MEMBRANES

3.1. Summary.

Binding sites for transcobalamin II-vitamin B_{12} complex from rat plasma were localised on isolated rat liver plasma membranes. The saturable binding process had an apparent affinity constant of 8.6×10^9 M^{-1}. The specific binding capacity of the plasma membranes was 1.5×10^{10} binding sites per mg membrane protein. No competitive inhibition was observed with free vitamin B_{12} or unsaturated trans-cobalamin II. A large part of the binding activity could be detached from the membrane either by incubation in detergent solution or by incubation in the presence of trypsin, whereas several other hydrolytic enzymes were found to be ineffective in this respect. The binding affinity of the plasma membranes prepared from other organs was of the same order of magnitude as the affinity of the binding sites from the liver.

3.2. Introduction.

The high association constant of vitamin B_{12} for its plasma transport protein, transcobalamin II (TC II), 2×10^{11} M^{-1} at body temperature (1), and the consequently low concentration of free vitamin B_{12} in the blood plasma, make it very unlikely that vitamin B_{12} enters the tissue cells in the free form. Transcobalamin II is thought to be a mediator in the process of uptake by the cells (2,3). This was confirmed by the observations on patients with congenital deficiency of TC II, who are unable to utilize

physiological amounts of vitamin B_{12} (4). The studies of
Pletsch and Coffey (5) demonstrated that in vivo the TC II-
vitamin B_{12} complex.binds to the surface membrane of the rat
liver cells prior to the cellular uptake. This uptake is
presumably mediated by pinocytosis, because the distribution
of the vitamin B_{12} molecules over the different cell compart-
ments showed a shift sequentially from plasma membranes to
microsomal vesicles, lysosomes and finally mitochondria. The
binding of TC II-vitamin B_{12} to the plasma membrane suggests
the presence of specific binding sites for TC II-vitamin B_{12}.
Such binding sites have been described earlier by Fiedler-
Nagy et al. (6). In this chapter these observations are
partially confirmed, and extended with data on the
characteristics of the binding process.

3.3. Methods.

3.3.1. Partial purification of transcobalamin II-vitamin B_{12}
       complex.

    Transcobalamin II- $\left[^{57}Co \right]$ CN-Cbl was purified from
normal rat serum as described in 2.3.1 but an additional
purification step was included, namely precipitation of the
serum with 60% $(NH_4)_2SO_4$, after saturation with $\left[^{57}Co \right]$ CN-
Cbl. The precipitated protein was dissolved in 20 mM Tris-
HCl pH 7.4 and further purified on Sephadex G 150 as
described in 2.3.1. The final purification factor was
increased to 300 times by pooling only the right part of
the TC II peak in the Sephadex G 150 eluate. The specific
binding activity amounted to 11.7 µg CN-Cbl/mg protein.

    The pooled material was concentrated by ultrafiltration
and dialysed against 20 mM Tris-HCl, 0.15 M NaCl pH 7.4.

3.3.2. Isolation of rat liver plasma membranes.

After exsanguination of the rats (200-250 g) by heart puncture the livers were removed, homogenized and centrifuged according to Neville (7) as modified by Ray (8), with the exception that the final density gradient purification step was performed in a Sorvall SZ-14 reorienting zonal rotor according to Evans (9). The zonal rotor was dynamically loaded with solutions of sucrose in 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.4 in the following order: 100 ml of 45%, a linear gradient of 600 ml from 45% to 31%, a linear gradient of 200 ml from 31% to 22.5%, 100 ml of 22.5% and 200 ml of 8% sucrose (w/w). The linear gradients were generated with a Sorvall GF-2 gradient maker. The sample was introduced in a volume of 25 ml and centrifugation was carried out at 4 000 rpm for 50 min at $5^{O}C$. At the end of the run the rotor was emptied statically by pumping the gradient from the bottom of the rotor, while the effluent was monitored for protein at 280 nm. 12 ml fractions were collected and the sucrose concentration of the fractions was determined by measurement of the refractive index.

The fractions rich in plasma membrane marker enzyme, 5'-nucleotidase, and poor in mitochondrial marker enzyme, succinate dehydrogenase, were pooled, diluted with one volume of 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.4 and collected by centrifugation for 30 min at 17 000 x g. The membranes were washed twice with bicarbonate, calciumchloride solution and finally resuspended to a concentration of 5 mg/ml. The purity of the isolated membranes was determined by measure- ment of the activities of 5'-nucleotidase succinate dehydro- genase, the lysosomal marker β-glucuronidase and of protein. Table 3.1 summarizes these analytical data, which are generally in agreement with data in the literature (8,9).

5'-nucleotidase was measured according to Persyn et al. (10), succinate dehydrogenase as described by King (11) and

β-glucuronidase by the method of Levvy et al. (12). Protein was measured according to Lowry et al. (13) with bovine serum albumin as a standard.

3.3.3. Membrane_binding_assay.

Binding studies were carried out at room temperature unless indicated otherwise. The standard incubation mixture contained a varying amount of rat TC II–$\left[^{57}Co\right]$ CN-Cbl, 147 μmoles NaCl, 4 μmoles KCl, 5 μmoles CaCl$_2$, 10 μmoles Tris-HCl, pH 7.2 and 0.5 mg of membrane protein in a volume of 1 ml. After the incubation, generally for 30 min, the mixtures were diluted with 3 ml of ice-cold 1 mM NaHCO$_3$, 0.5 mM CaCl$_2$, pH 7.4, collected by centrifugation for 10 min at 3 500 x g at 4°C and washed twice with the same solution.

The radioactivity in the pellet was measured and calculated as pmoles $\left[^{57}Co\right]$ CN-Cbl by comparison with $\left[^{57}Co\right]$ CN-Cbl standard solutions of known specific activity. All incubations were carried out in duplicate and correction for non-specific binding to the assay materials was obtained by incubations without plasma membrane material.

3.4. Results.

3.4.1. Localization_of_the_membrane_binding_sites.

An initial experiment was carried out to demonstrate that TC II–$\left[^{57}Co\right]$CN-Cbl binds exclusively to the plasma membrane. An unfractionated 1 000 x g sediment from rat liver homogenate was incubated in a standard incubation mixture with TC II–$\left[^{57}Co\right]$CN-Cbl and separated in a zonal sucrose density gradient as described in 3.3.2. The separate fractions were analysed for radioactivity, sucrose density,
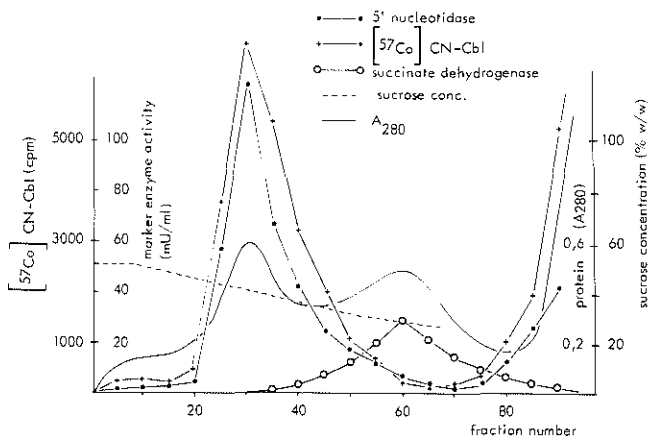
fig. 3.1. *Distribution pattern after sucrose density gradient centrifugation of a 1 000 x g sediment from rat liver homogenate, which was preincubated with partially purified TC II-$\left[^{57}Co\right]CN$-Cbl.*

5'-nucleotidase and succinate dehydrogenase activity. As demonstrated in figure 3.1 the $\left[^{57}Co\right]$CN-Cbl radioactivity and 5'-nucleotidase activity coincided at sucrose concentrations from 37-41%. Peak activities were located at 39% sucrose, which has been formerly shown to correlate with the density of plasma membranes (8). These data confirm that binding sites for the TC II-vitamin B_{12} complex are present on the rat liver plasma membranes.

Table 3.1. *Analytic data of plasma membrane preparations.*

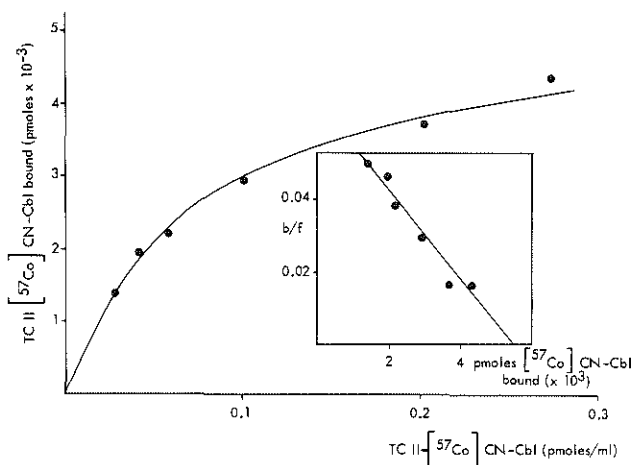| assay	result as a mean of 3 experiments
milligrams of protein per g liver, wet weight	1.44
5'-nucleotidase	505 mU/mg protein
succinate dehydrogenase	2,5 mU/mg protein
β-glucuronidase	0 mU/mg protein
recovery based on 5'-nucleotidase	20 %
enrichment based on 5'-nucleotidase	21-fold

fig. 3.2. Binding of TC II-[⁵⁷Co]CN-Cbl to plasma membranes
         as a function of TC II-CN-Cbl concentration. 0.5
         mg of membrane protein was incubated in the
         standard incubation mixture for 30 min at 25°C at
         different TC II-[⁵⁷Co]CN-Cbl concentrations.
         Insert: Scatchard plot of the experimental data.
         The intercept on the abscissa represents the
         maximal amount of TC II-[⁵⁷Co]CN-Cbl bound or of
         available binding sites, whereas the slope of the
         curve stands for the apparent affinity constant.

3.4.2. Binding of transcobalamin II-vitamin B_{12} to isolated
       plasma membranes.

     In 5 separate experiments, in which plasma membrane
fractions from different liver homogenates were used, the
membrane binding capacity for TC II-vitamin B_{12} was studied
as a function of the concentration of TC II-vitamin B_{12}. The
binding was saturable and an estimate of the apparent
affinity constant was made using Scatchard analysis, in
which the "bound to free" ratio is plotted versus the
amount of TC II-[⁵⁷Co]CN-Cbl bound (figure 3.2. + insert).
The affinity constant, represented by the slope of the line,
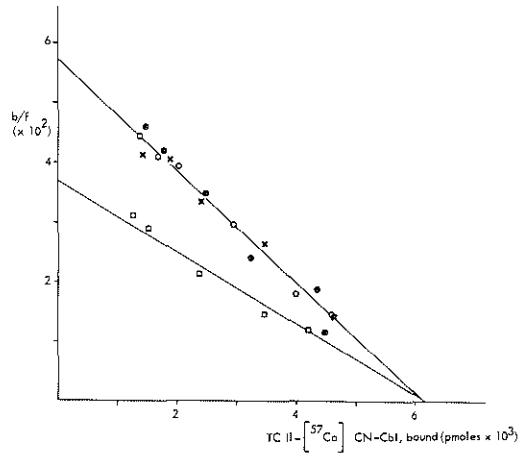ranged from 5 to 12 x 10^9 M^{-1} with a mean of 8.6 x 10^9 M^{-1}

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fig. 3.3. The influence of free CN-Cbl, unsaturated and
CN-Cbl-saturated plasma on the binding of TC II-
$[^{57}Co]$ CN-Cbl to plasma membranes. The conditions
were the same as in fig. 3.2. ○——○, standard
incubation; x——x, with 0.2 pmoles CN-Cbl;
●——●, with 0.2 ml unsaturated plasma and
□——□, with 0.2 ml saturated plasma.

(n=5). The specific number of binding sites was calculated
from the intersections on the abscissa of the Scatchard plot
and ranged from 0.6 to 3.7 x 10^{10} (mean 1.5 x 10^{10}) per mg of
membrane protein. The specificity of the binding process
with respect to free CN-Cbl and saturated or unsaturated
binding protein is illustrated in figure 3.3. Scatchard
analysis of the binding in the presence of free CN-Cbl or
unsaturated rat plasma indicated that no competition between
the labeled saturated binder and the free vitamin or
unsaturated TC II took place in contrast with unlabeled
saturated TC II, which reduced the affinity for TC II-$[^{57}Co]$
CN-Cbl. These data suggest that the plasma membrane binding
sites have a clear preference for saturated TC II in

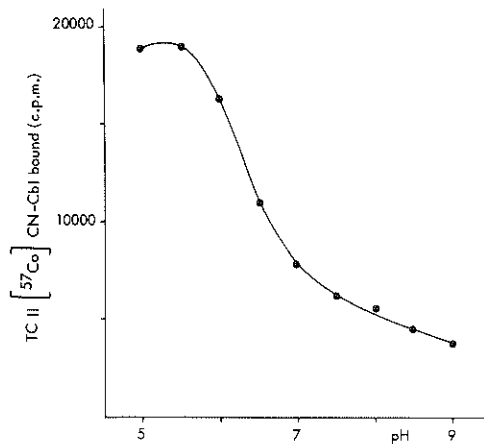*Fig. 3.4. pH dependence of the binding process. The incubation medium contained 50 mM Tris-maleate buffer, 4 mM CaCl$_2$, 0.07 pmoles TC II-[^{57}Co] CN-Cbl and 0.6 mg of plasma membrane protein in 1 ml.*

comparison to free CN-Cbl and unsaturated binding protein in the conditions used in our experiments.

The binding of free [^{57}Co] CN-Cbl to the isolated membranes was about 8% of the binding of the TC II-CN-Cbl complex, which agrees well with the findings of Fiedler-Nagy et al. (6).

3.4.3. Effects of calcium, EDTA, pH and temperature.

The binding of TC II-CN-Cbl to liver plasma membranes was not influenced by the presence or absence of calcium ions. Addition of EDTA (2.5 mM) instead of calcium caused in different membrane preparations, a variable increase in the maximal amount of bound TC II-CN-Cbl, without affecting the affinity constant. The highest number of binding sites, observed in the presence of EDTA, was 6.6 x 10^{10} per mg of
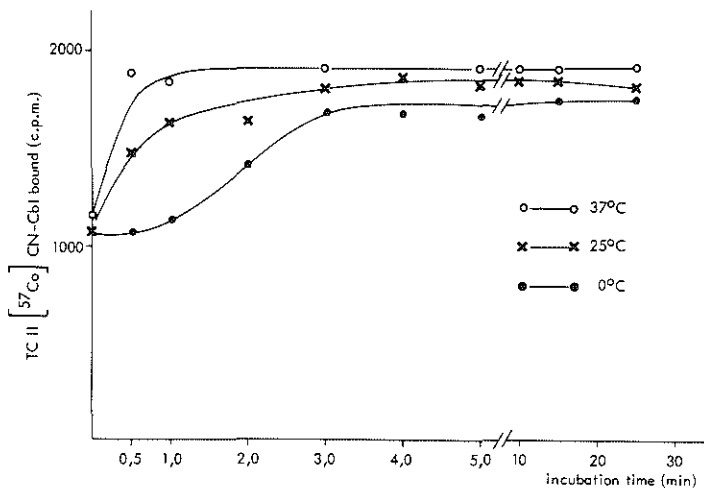
Fig. 3.5. Time and temperature dependence of the binding process. 0.3 mg of membrane protein was incubated in the standard incubation medium with 0.07 pmoles TC II-$\left[^{57}Co\right]$ CN-Cbl per ml at three different temperatures.

membrane protein.

The pH dependence of the binding process is shown in figure 3.4. Maximal binding occurred at pH 5.5 and a steady decrease took place to a minimum at pH 9.0.

An analysis of the time dependence of the binding process is given in figure 3.5. Although the rate at which equilibrium was reached, increased with temperature, the level of the plateau was the same at all temperatures and was maintained for at least 30 min.

3.4.4. Effects of enzymes, detergents and reducing agents.

Isolated plasma membranes were treated with several hydrolytic enzymes or detergents, washed thoroughly and incubated with TC II-$\left[^{57}Co\right]$ CN-Cbl to measure the effects

Table 3.2. Effect of enzyme incubations, detergents and
SH-reagents on the binding of TC II-[^{57}Co] CN-Cbl
by liver plasma membranes.

treatment/additive	concentration	percentage of control bound
none		100 %
neuraminidase	0,25 mg/ml	110 %
lipase	0,50 mg/ml	97 %
mannosidase	0,25 mg/ml	100 %
β-galactosidase	0,25 mg/ml	100 %
trypsin	0,50 mg/ml	30 %
phospholipase A	0,50 mg/ml	95 %
triton X-100	1 %	40 %
sodium deoxycholate	1 %	35 %
dithiothreitol	2,5 mM	98 %
2-mercaptoethanol	2,5 mM	100 %

*Membrane material was preincubated with the enzymes for
60 min at 37°C in 0.15 M NaCl or, with neuraminidase, in
0.2 M sodium acetate buffer pH 5.6, washed two times with
1 mM NaHCO$_3$, 0.5 mM CaCl$_2$, pH 7.4 and suspended in the same
volume as the control. The same procedure was followed in
case the membranes were incubated with detergents. The
standard incubation mixture contained 0.6 mg of membrane
protein and 0.1 pmole of rat TC II-[^{57}Co] CN-Cbl per ml.
The effects of dithiothreitol and 2-mercaptoethanol were
studied in the standard incubation mixture. The enzymes
were purchased from Boehringer (Mannheim, Germany).*

of the pretreatment on the binding process. The results are given in table 3.2.

Only trypsin and the two detergents reduced the amount of bound radioactivity significantly. Incubations with different TC II-$[^{57}Co]$ CN-Cbl concentrations showed that the reduction in binding was not due to a decrease in binding affinity but to a reduction in the number of binding sites.

2-mercaptoethanol and dithiothreitol did not influence the binding when present in the standard incubation mixture. This suggests that for the binding mechanism intact disulfide bridges are not essential.

3.4.5. Binding to rat organ tissues.

Plasma membrane-rich 1 000 x g subfractions of homogenates from heart, liver, brain, kidney and spleen were prepared and the binding affinity for TC II-CN-Cbl was studied. The respective affinity constants, obtained by Scatchard analysis, are presented in table 3.3.

Table 3.3. Binding affinity of plasma membrane-rich fractions from various rat tissues.

tissue	affinity constant
liver	$4.5 \times 10^9 M^{-1}$
heart	$4.0 \times 10^9 M^{-1}$
brain	$4.7 \times 10^9 M^{-1}$
kidney	$3.7 \times 10^9 M^{-1}$
spleen	$4.0 \times 10^9 M^{-1}$

3.5. Discussion.

Our studies demonstrate the presence of binding sites
for transcobalamin II-CN-Cbl on the plasma membranes of rat
liver cells. This finding confirms the assumption of the
presence of such binding sites, made by other investigators
on the basis of vitamin B_{12} transport studies in intact
animals and cell suspensions (5,14). In our studies the
plasma membrane fraction was prepared from a low centrifugal
force pellet from a rat liver homogenate according to Evans
et al. (9).

Fiedler-Nagy et al.(6), who isolated membranes from
a microsomal pellet, studied the membrane affinity towards
TC II-vitamin B_{12} in unfractionated plasma. Our data are in
agreement with their results regarding the affinity constant
of the binding, but our calculation of the number of specific
binding sites is somewhat lower. A major difference with the
studies of Fiedler-Nagy et al. (6) concerns their finding
that plasma, not saturated with vitamin B_{12}, inhibited the
uptake of radioactive vitamin B_{12} bound to transcobalamin.
On the contrary, in the present study no inhibition of the
binding by free CN-Cbl or unsaturated plasma was found,
which may be explained by the fact that plasma and the TC II-
$\left[^{57}\text{Co} \right]$ CN-Cbl were added at the same time, whereas Fiedler-
Nagy et al. (6) pre-incubated the plasma membrane with the
unsaturated plasma. Physiologically it seems unlikely that
unsaturated TC II competes with TC II-CN-Cbl for the
binding sites on the cell membrane, because in vivo the
higher level of unsaturated TC II in blood plasma would
prevent the attachment of saturated TC II to the binding
sites and the subsequent uptake into the cells.

Accordingly, Hall has reported recently that Hela cells
and human lymphocytes showed a preferential uptake of
saturated TC II, which was not affected by a 6-fold excess
of saturated binder (15).

The pH optimum of 5.5 for rat liver membranes is the same as for the primary uptake of TC II-vitamin B_{12} by mouse leukemia cells (14). Liver membranes, remarkably, do not require the presence of Ca^{++} for the binding process and EDTA even seems to render more binding sites accessible for the TC II-vitamin B_{12} complex. On the contrary the binding and uptake by several other cell types (2,3,14,16) is strongly inhibited by EDTA. These observations suggest that the liver membrane binding sites differ from the binding sites on other cells.

In an attempt to define the nature of the membrane binding sites by treatment of the purified membranes with several hydrolytic enzymes, and measurement of the effect on the binding activity, only trypsin proved to reduce the number, but not the affinity, of the binding sites. One may conclude from this, that at least a part of the binding site contains protein. Sialic acid does not seem to be directly involved because neuraminidase had no effect on the binding process. Lipolytic enzymes also did not interfere with the binding activities of the membranes in our experiments.

The results of this study demonstrate, that a protein-containing compound from liver plasma membrane specifically binds TC II-vitamin B_{12} probably as a primary step in the uptake mechanism. Data from kidney and liver uptake of TC II-vitamin B_{12} suggest that after a pinocytotic process the complex moves into the lysosomal system, where it is probably degraded and the vitamin released from its binding protein (5,17,18). If the binding sites are localized on those parts of the membrane which are subject to pinocytosis, a continuous flow of TC II-vitamin B_{12} into the cell can be expected, also in view of the affinity constant, which indicates that at a normal TC II-vitamin B_{12} concentration in the plasma of about 500 pM, most binding sites will be occupied by TC II-vitamin B_{12}.

The binding affinities of membrane preparations from

different rat tissues are all of the same order of magnitude. This indicates that possible differences in the rate of uptake are likely to be the result of differences in the number of receptor sites on the membrane or in the velocity with which the pinocytotic process transports the complex into the cell.

3.6. References.

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BINDING, UPTAKE AND RELEASE OF TRANSCOBALAMIN II-$[^{57}Co]$ CYANOCOBALAMIN BY ISOLATED RAT LIVER PARENCHYMAL CELLS

4.1. Summary.

Isolated rat liver parenchymal cells were incubated with partially purified rat or human TC II-vitamin B_{12}, which was radioactively labeled in the vitamin moiety. A rapid binding onto the surface of the cells in the first few minutes was followed by a steady linear increase of intra-cellular $[^{57}Co]$ CN-Cbl in the next hour. With increasing concentrations of TC II the uptake became partially saturated and apparently TC II-Cbl is taken by two processes, a saturable specific uptake and a non-saturable non-specific uptake. The former process is probably due to specific binding sites on the plasma membrane. In contrast to observations on other cells, the process of binding and uptake was independent of calcium and could not be inhibited by EDTA or EGTA. Chloroquine, a lysosomotropic agent, caused an accumulation of intracellular Cbl, presumably by inhibition of intralysosomal proteolysis of the transport protein. It was concluded first, that TC II-vitamin B_{12} is taken up by a mechanism of adsorptive endocytosis, followed by fusion with the lysosomes, and second that normally the cells secrete most of the internalized vitamin shortly after the uptake. This was confirmed by the observation that 30-100% of radioactive vitamin B_{12}, which had been taken up shortly before the isolation of the liver cells, was released again in two hours of incubation.

4.2. Introduction.

In chapter 3 the binding of the TC II-vitamin B_{12} complex to isolated plasma membranes has been described and it was concluded that the first step in the entrance of vitamin B_{12} into the cell is the binding of TC II-vitamin B_{12} to specific binding sites on the plasma membrane. The progress of the uptake mechanism, however, remains to be studied. Several mechanisms are possible: the carrier protein stays behind while the vitamin is transported through the membrane into the cell, or the whole complex enters the cytoplasm through the membrane or the complex enters the cell engulfed in pinocytotic vesicles (1).

As a logical sequel to the liver plasma membrane experiments the isolated liver cell was chosen as an in vitro system to study the uptake of vitamin B_{12} by the cell. Liver parenchymal cells are rather easy to isolate, can be maintained in suspension in a relatively normal, viable state (2) and have been used previously for the investigations of the uptake and degradation of rat high-density lipoprotein (3) and asialoglycoprotein (4).

In this chapter the results of experiments are presented in which the vitamin moiety is radioactively labeled. Some of the experiments were carried out with human transcobalamin II and some with rat TC II for practical reasons and also to establish that there was no fundamental difference in the uptake of vitamin B_{12}, whether it was bound to human or to rat TC II.

4.3. Methods.

4.3.1. Preparation of the liver cell suspension.

Liver parenchymal cells were isolated as described in

section 2.4. The cells were washed and finally suspended in
Dulbecco's modified Minimal Essential Medium (DMEM) in a
concentration of 3.6 x 10^6 cells/ml. All glassware was
siliconized in 1% siliclad (Clay-Adams), rinsed with
distilled water and dried.

4.3.2. Preparation of transcobalamin II-$[^{57}Co]$ cyanocobalamin.

TC II-$[^{57}Co]$ CN-Cbl was partially purified as described
in section 2.3.1. The final preparation was dialyzed against
2 x 1 L DMEM for 48 hours and centrifuged at 100 000 x g for
1 hour. All necessary dilutions were made in DMEM with
0.09% human serum albumin and buffered at pH 7.4 with 25 mM
$NaHCO_3$.

4.3.3. Standard incubation procedure.

7 ml aliquots of a cell suspension were distributed
over 25 ml Erlenmeyer flasks and kept on melting ice. Just
before the incubation the flasks were preincubated in a
shaking waterbath at 37° for 5 min. The incubation was
started by the transfer of 5 ml cell suspension to another
25 ml Erlenmeyer, which contained TC II-$[^{57}Co]$ CN-Cbl and
eventually other additives in a volume of 4 ml. These latter
flasks were also preincubated at $37^O C$. The Erlenmeyers were
placed in a shaking waterbath and gassed with 95% O_2, 5% CO_2.
At various time intervals 0.5 ml duplicate samples were
taken and the cells were separated from the medium with the
silicon oil filtration-centrifugation technique described
in section 2.5. The cellular uptake was corrected for
adherent waterspace as outlined in the same section and
expressed as pmoles/10^9 cells.
To avoid contact of the cells with too high concentra-

tions of the agents, EDTA, EGTA were added in a concentration
which gave an excess of 1 mM over the concentration of biva-
lent cations in the medium. This excess was checked by
complexometric titration with eriochrome black T. It was
taken care off that the normal pH of the medium was
maintained after the sequestration of the cations.

4.4. Results.

4.4.1. Dependence of binding and uptake on concentration of
TC II-[^{57}Co]CN-Cbl.

Binding and uptake of rat TC II-[^{57}Co]CN-Cbl by the
isolated liver parenchymal cells showed a rapid rise in the
first 5 min and a steady linear increase in the next hour.
(fig. 4.1.). The first phase is thought to represent the
adherence of the protein to the binding sites on the plasma
membrane and the second phase probably represents the
entrance of the complex into the cells. Rat TC II and human
TC II (figure 4.2) showed corresponding patterns, but human
TC II seemed to be bound and taken up in somewhat higher
amounts. The amount of initial binding and the rate of
uptake were dependent on the extracellular concentration of
TC II.

The initial binding was defined as the intercept on the
ordinate, which is obtained by extrapolation of the time
dependent uptake curve to time zero. In figure 4.3 the
binding of rat TC II-[^{57}Co] vitamin B_{12} is plotted as a
function of the concentration and figure 4.4 shows the rate
of uptake, represented by the slope of the time dependent
uptake curve from 5 to 60 min, as a function of the
concentration. Both curves do not seem to reach saturation
but the profile of the curves suggests that the measured
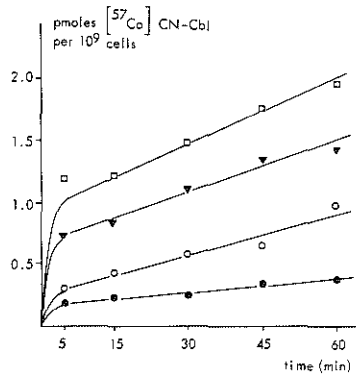binding and uptake are the sum of a saturable and a non-

fig. 4.1. Time course of the binding and uptake of rat TC II-
$[^{57}Co]$ *CN-Cbl by rat liver parenchymal cells at*
four concentrations: ● ──── ●,*70 pM;* ○ ──── ○,*200 pM;*
▼ ──── ▼,*400 pM and* □ ──── □,*680 pM TC II-*$[^{57}Co]$*CN-Cbl.*
Each point is the mean of duplicate measurements.

saturable component.

The saturable component is probably the result of
binding of TC II-vitamin B_{12} to the specific binding sites
on the plasma membrane, whereas the non-saturable uptake
is probably caused by non-specific binding to the cell
membrane.

With human TC II-$[^{57}Co]$ CN-Cbl we were able to analyse
this non-specific binding and uptake by incubations in the
presence of 10 nM excess unlabeled human TC II-CN-Cbl. At
this high concentration and consequently low specific
radioactivity of the TC II-CN-Cbl the measured specific
binding and uptake of $[^{57}Co]$ vitamin B_{12} is negligible in
relation to the total binding and uptake. The observed rise
in cellular radioactivity with time was therefore assumed
to represent largely non-saturable and non-specific uptake.

The non-specific binding of human TC II-$[^{57}Co]$ CN-Cbl

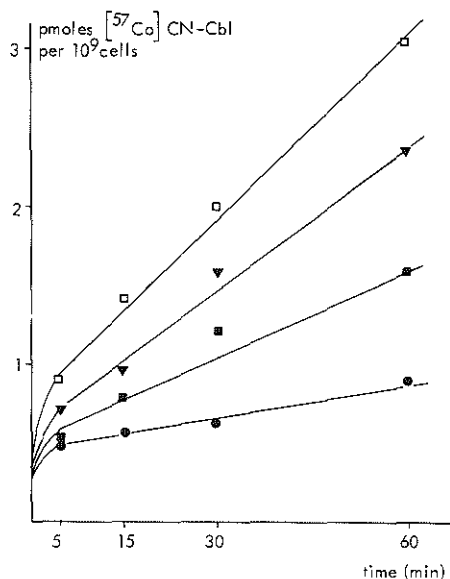fig. 4.2. *Time course of the binding and uptake of human*
TC II-$\left[^{57}Co\right]$ CN-Cbl by rat liver parenchymal cells
at four concentrations: ●——●,*95 pM;* ■——■,*191 pM;*
▼——▼,*395 pM and* □——□,*615 pM TC II-$\left[^{57}Co\right]$ CN-Cbl.*
Each point is the mean of duplicate measurements.

in the presence of 10 nM TC II-CN-Cbl was a linear function
of the concentration of TC II-$\left[^{57}Co\right]$ CN-Cbl. The specific
binding was calculated by subtraction of the non-specific
binding from the partially saturable total binding observed
in the absence of unlabeled TC II-CN-Cbl. The specific
binding plotted as a function of the concentration (figure
4.5) was saturable and had an affinity constant of 6.9 x 10^9
M^{-1}, calculated by Scatchard analysis.

A similar procedure was followed for the kinetic
analysis of the rate of uptake as a function of the
concentration. The rates of uptake at each concentration of
TC II-$\left[^{57}Co\right]$ CN-Cbl in the presence of 10 nM unlabeled TC II-
CN-Cbl were subtracted from the rates of uptake at the same
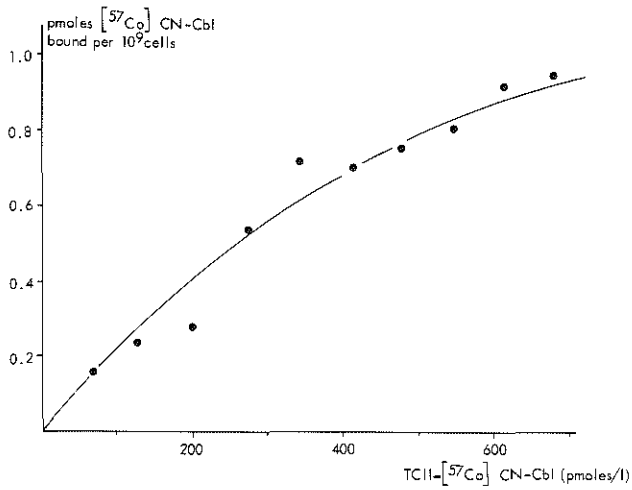concentrations without unlabeled TC II-CN-Cbl. The rate

fig. 4.3. Binding of $[^{57}Co]$ CN-Cbl to liver parenchymal
cells as a function of the rat TC II-$[^{57}Co]$ CN-Cbl
concentration. Each point represents the amount of
cell associated radioactivity at time zero, obtained
by extrapolation of the linear part of the curves
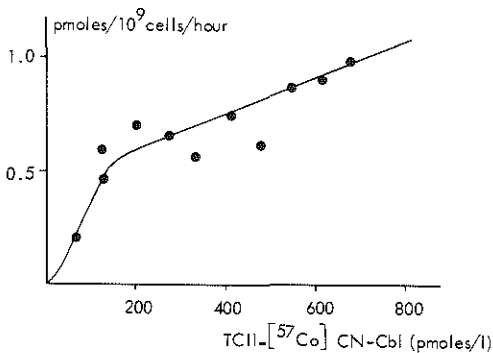in figure 4.1, at the indicated concentration.

Fig. 4.4. Rates of uptake of $[^{57}Co]$ CN-Cbl as a function of
the rat TC II concentration. Each point represents
the mean rate of uptake from 5 to 60 min at the
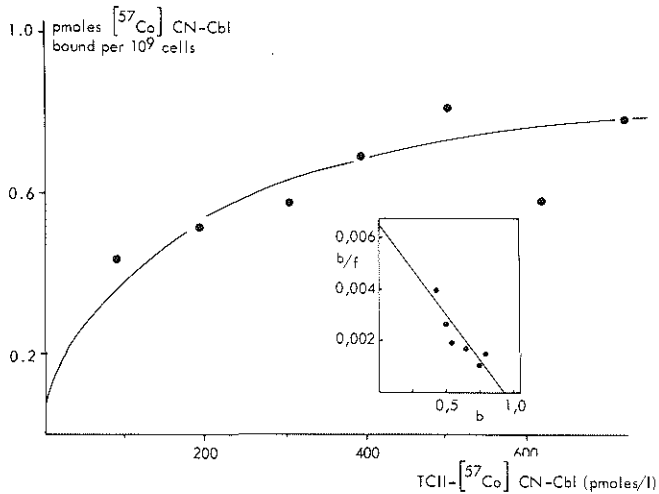indicated concentration.

fig. 4.5. Specific binding of $\left[^{57}Co\right]$ CN-Cbl to liver parenchymal cells as a function of the human TC II-$\left[^{57}Co\right]$ CN-Cbl concentration. Each point is obtained by subtraction of the non-specific binding of $\left[^{57}Co\right]$ CN-Cbl from the total binding of $\left[^{57}Co\right]$ CN-Cbl at the indicated concentration. The insert represents the Scatchard plot of the same experimental data.

of non-specific uptake was a linear function of the concentration but the calculated rate of specific uptake was saturated at about 400 pM and reached a maximal value of 1.1 pmoles/10^9 cells/hour (figure 4.6). Half-maximal rate of specific uptake was reached at about 140 pM which is close to the concentration at which half-maximum binding occurred, namely 145 pM.

4.4.2. Role of calcium in the TC II-Cbl uptake.

The effect of EDTA and EGTA on the binding and uptake was studied at 100 and 600 pM of TC II-$\left[^{57}Co\right]$ CN-Cbl. At both concentrations binding and uptake were equal to the
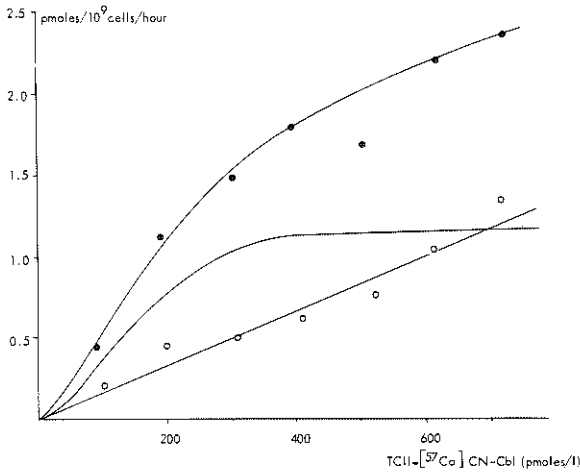
fig. 4.6. *Rates of uptake of* $\left[^{57}Co\right]$ *CN-Cbl as a function of the human TC II-*$\left[^{57}Co\right]$ *CN-Cbl concentration. Each point represents the mean rate of uptake from 5 to 60 min at the indicated concentration.* ●————●, *incubation with TC II-*$\left[^{57}Co\right]$ *CN-Cbl alone;* ○————○, *incubation with TC II-*$\left[^{57}Co\right]$ *CN-Cbl in the presence of 10 nM pure human TC II-CN-Cbl;* ————,*course of the rate of specific uptake, obtained by subtraction of the lower curve from the top curve.*

control incubations during the first 30 min. After this time the liver cells tended to agglutinate in the presence of the chelators, which resulted sometimes in large differences in the duplicate measurements. However, the conclusion seems to be justified that the binding and uptake of TC II by liver parenchymal cells are calcium and/or magnesium independent. This is in agreement with the observations on the binding of TC II-Cbl to isolated liver plasma membranes which was also no inhibited by the addition of EDTA or EGTA (chapter 3).
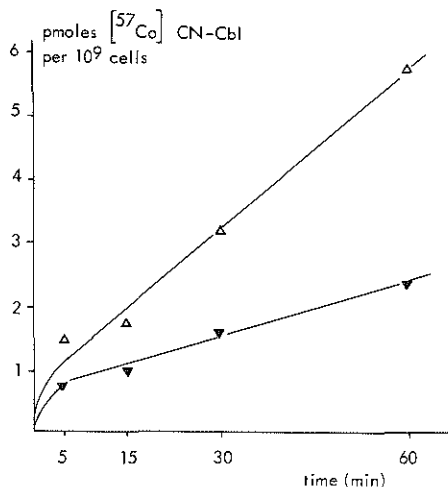
fig. 4.7. *Effect of chloroquine on the binding and uptake*
of human TC II-$\begin{bmatrix} 5^7 Co \end{bmatrix}$ CN-Cbl. ▼———▼,control cells,
incubated with 400 pM TC II-$\begin{bmatrix} 5^7 Co \end{bmatrix}$ CN-Cbl.
△———△,cells incubated with 400 pM TC II-$\begin{bmatrix} 5^7 Co \end{bmatrix}$
CN-Cbl and 3.5 mM chloroquine.

4.4.3. Effect of chloroquine on the uptake of TC II-$\left[^{57} Co \right]$ CN-Cbl.

The data in section 4.4.1 indicated that TC II-CN-Cbl
is bound to the cell membrane and subsequently is taken up
by the cells. It was, however, uncertain whether the measured
uptake represented the total uptake by the cells or whether
it was the resultant of an uptake and a release process in
which the uptake was somewhat larger than the release. The
latter concept was supported by the results of an experiment
in which chloroquine was added to the incubation medium.
This lysosomotropic agent, an inhibitor of lysosomal
proteolysis, caused an almost threefold increase of the
cellular uptake without affecting the initial binding of
TC II-CN-Cbl to the cell membrane. (figure 4.7). Vitamin B_{12}
apparently accumulates in the lysosomes as a result of the

inhibition of the proteolytic degradation of TC II. This observation also indicates that in the absence of chloroquine the real rate of uptake is higher than the observed rate of uptake and that a part of the internalized vitamin B_{12} is released by the cells shortly after the uptake.

4.4.4. Release of $[^{57}Co]$ Cbl from isolated liver cells.

The data from the experiments with chloroquine suggest that Cbl is released from the cells shortly after the uptake. In order to measure this release, experiments were carried out, in which the liver cells were loaded with radioactively labeled vitamin B_{12} by the intravenous injection of $[^{57}Co]$ CN-Cbl, prior to the isolation procedure. After isolation of the parenchymal cells the release of the radioactive label was measured during incubations under standard conditions.

When the injection of $[^{57}Co]$ CN-Cbl took place 30' before the start of the liver cell isolation, a substantial part of the radioactive vitamin B_{12} was released into the medium (figure 4.8). The initial release became larger with increasing extracellular TC II concentrations, but the subsequent releases were apparently not influenced by the extracellular TC II concentrations. Without extracellular TC II about 15% of the cellular radioactivity was released within the first minute but at 280 pM TC II almost 50% could be recovered instantaniously in the medium. At this high concentration of TC II the total cellular content of $[^{57}Co]$ Cbl was released in two hours. At $0^{o}C$ some initial release occurred but no further loss of radioactivity was detectable in the following two hours.

In contrast with the release of recently internalized radioactive Cbl, the total release of endogenous vitamin B_{12}, measured with the radioassay, increased linearly and independently of the extracellular concentration of TC II,
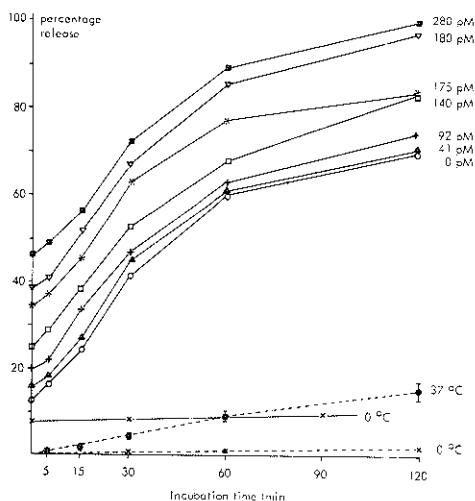
fig. 4.8. *Release of $[^{57}Co]$ CN-Cbl and unlabeled Cbl from
isolated liver cells, expressed as percentage of
the initial cellular amount of $[^{57}Co]$ CN-Cbl and
Cbl. The cells were loaded with radioactive
vitamin B_{12} by an intravenous injection of 1 μCi
$[^{57}Co]$ CN-Cbl 30 min before the isolation of the
parenchymal cells. The loaded cells were incubated
under standard conditions with partially purified
rat TC II-CN-Cbl in concentrations ranging from
0 to 280 pM. The solid lines represent the release
of $[^{57}Co]$ CN-Cbl at the various concentrations of
unlabeled TC II-Cbl and each point is the mean of
duplicate measurements. The broken lines represent
the release of total unlabeled Cbl and each point
represents the mean value ± S.D. of the incubations
at all different TC II-Cbl concentrations. In
addition the release of Cbl and $[^{57}Co]$ CN-Cbl was
measured at $0^{o}C$: x———x, release of $[^{57}Co]$ CN-Cbl;
x------x, release of total unlabeled Cbl.*

(figure 4.8 broken lines). It reached only 15% of the amount
of total initial intracellular vitamin B_{12} content. At $0^{o}C$

release of endogenous vitamin B_{12} was absent. It is possible
that a part of the released vitamin originated from cells
which became leaky during the incubation, but comparison
with the activity of the cytoplasmic marker enzyme LDH in
the medium showed that the percentage of release of vitamin B_{12}
was at least two times higher than the percentage of release
of the enzyme (see section 2.4). Therefore part of the
observed vitamin B_{12} release was explained as an active
release from the cells.

When radioactive vitamin B_{12} was injected 70 hours
before the onset of the liver cell isolation, the initial
$[^{57}Co]$ vitamin B_{12} release was negligible at all extracellu-
lar concentrations of TC II-CN-Cbl and also the further
release was independent of the extracellular TC II
concentration. The release was linear and reached only 18.6%
of the cellular radioactivity after 2 hours of incubation.
The release of total endogenous vitamin B_{12} in this experi-
ment was 19.3% after 2 hours of incubation and it can be
concluded that after 70 hours the radioactive vitamin B_{12}
has been taken up in the stable pool of endogenous vitamin
B_{12} and therefore is released in the same way as the
unlabeled vitamin in this pool.

4.4.5. Measurement of the excretion of vitamin B_{12} into the
bile.

In the preceding section it was demonstrated that
vitamin B_{12} is released from the liver parenchymal cells
at a relatively fast rate with respect to vitamin B_{12} in a
pool of recently internalized vitamin, and at a proportional-
ly slower rate for the large cellular pool. This release may
have occurred at the circulatory and/or the biliary side of
the liver cell.

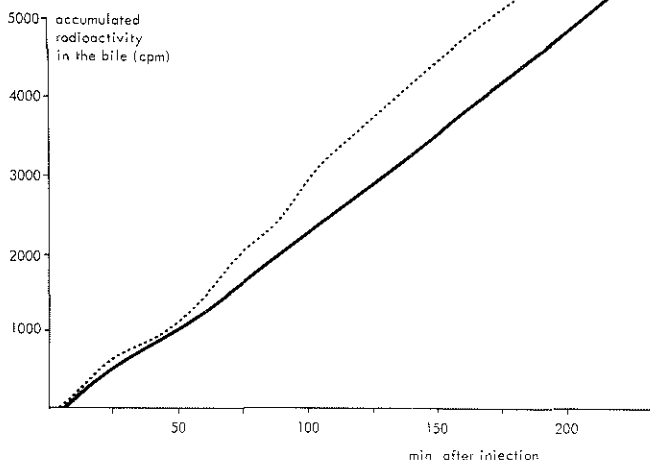In order to investigate the direction of the release

fig. 4.9. Accumulated radioactivity in the bile in two
         different rats after the intravenous administration
         of 1 μCi [^{57}Co] CN-Cbl.

of vitamin B_{12}, rats were intravenously injected with [^{57}Co]
CN-Cbl after insertion of a cannula into the bile duct. Bile
secretion remained constant at a flow of 0.3 to 0.4 ml/hour.
^{57}Co-radioactivity in the bile appeared shortly after the
injection and increased linearly during about 3 hours
(figure 4.9). The amount of radioactivity excreted after two
hours was 2-3% of the total liver radioactivity at that
moment. The total amount of vitamin B_{12} in the bile was
2.3 ng/ml, which is less than 0.1% per hour in relation to
the total liver vitamin B_{12} pool. The vitamin B_{12} in the
bile was in the free form as was established by Sephadex G50
column chromatography.

4.5. Discussion.

     The uptake of TC II-vitamin B_{12} by isolated liver cells

has three aspects: the binding of the complex to the specific binding sites on the plasma membrane, the transport into the cell, and the intracellular processing of the vitamin and the transport protein.

The biphasic profile of the time dependent increase in cell bound radioactivity suggests that the binding of TC II-Cbl to the surface of the cell predominates in the first phase of rapid rise in cell-associated radioactivity and that the further increase is the result of the entrance of the complex into the cell. It is difficult to measure binding and uptake separately and therefore the amount of binding at each concentration of TC II was estimated by extrapolation of the linear part of the curve, representing the increase of intracellular TC II-Cbl, to time zero.

The binding and uptake of human and rat TC II-Cbl by the liver cell were both partially saturable. It was assumed that a part of the process was non-specific and the size of the non-specific component was estimated by incubations in the presence of 10 nM unlabeled pure human TC II-CN-Cbl. The non-specific binding and uptake were a linear function of the concentration of TC II-$[^{57}Co]$CN-Cbl. The specific binding and uptake were calculated by subtraction of the non-specific binding and uptake from the total binding and uptake and were found to be saturable at concentrations above 400 pM. These results are in agreement with the observations of Youngdahl-Turner et al. (5) on the specificity of the binding of TC II-Cbl to fibroblasts in culture. The observed affinity constant for the binding of TC II-vitamin B_{12} by the liver cell is in agreement with the affinity constant which was found with the isolated liver plasma membranes. The similarity between the concentration dependent curves for binding and uptake suggests that the rate of uptake is primarily deter-mined by the degree of occupation of the specific binding sites. Further studies are necessary to find out to what extent the non-specific binding and uptake are physiological

phenomena or features of this particular liver cell system.

The observation that the binding and uptake of TC II by the liver cell is independent of calcium, confirms our results obtained with the binding of TC II to isolated liver plasma membranes and is also in agreement with the studies of Fiedler-Nagy et al. (6).

The accumulation of [57]Co-radioactivity in the cell as a result of the presence of chloroquine is explained by the fact that this lysosomotropic agent is an inhibitor of lysosomal proteolysis (7,8). Apparently TC II-vitamin B_{12} is transported to the lysosomes, where in the absence of chloroquine the protein is degraded and the vitamin B_{12} molecule is dissociated from its transport protein. A blockade of lysosomal proteolysis by chloroquine apparently prevents the bound cobalamin to be processed further by the cells, and causes an accumulation of cobalamin in the lysosomes and possibly the endocytic vesicles.

The combination of specific binding sites on the plasma membranes and the localization of TC II-vitamin B_{12} in the lysosomes after its internalization by the cell leads to the conclusion that the complex probably enters the cell by a process of binding site-mediated pinocytosis or adsorptive endocytosis (9).

Chloroquine does not affect the binding of TC II to the membrane binding sites and it is assumed that it does not influence the rate of endocytosis. Therefore the conclusion can be drawn that in the absence of chloroquine the real uptake of TC II-vitamin B_{12} is much higher than the observed uptake and that a part of the internalized vitamin B_{12} is released from the cells shortly after its uptake.

This release of vitamin B_{12} was studied directly by incubation of isolated liver cells from rats which had been intravenously injected with $\begin{bmatrix} 57 \\ Co \end{bmatrix}$ vitamin B_{12} before the isolation procedure was started. When the injection was given shortly before the isolation of the cells the total amount

of radioactivity could be released from the cells within 2 hours of incubation. Apparently there was still TC II-$[^{57}Co]$ vitamin B_{12} attached to the binding sites, because a part of the radioactive label was instantaneously released from the cells. This early release increased with the concentration of added extracellular TC II but the subsequent release of cell-associated radioactivity was independent of the extracellular TC II concentration. The latter radioactivity was probably located in endocytotic vesicles, or the lysosomes. Measurement of the total release of vitamin B_{12} revealed that there is a more stable pool of vitamin B_{12} in the cell from which vitamin B_{12} is released steadily. The release of this endogenous vitamin B_{12} in absolute quantities exceeded the release of radioactive vitamin B_{12} but it amounted to only 15% of the total liver vitamin B_{12} content. Moreover, it was independent of extra-cellular TC II concentrations. When $[^{57}Co]$ CN-Cbl was administered 70 hours before the cell isolation, the release of ^{57}Co-radioactivity was proportional with the total vitamin B_{12} secretion, which means that in 70 hours the $[^{57}Co]$ vitamin B_{12} was fully integrated into the stable pool.

Our data on the release of vitamin B_{12} from isolated liver cells correspond with results from studies by Cooksley et al. (10,11), who observed releases of vitamin B_{12} by the perfused rat liver in vivo during 4 hours of 15%, 30% or 87% after the liver had been labeled with $[^{57}Co]$ CN-Cbl 96 hours, 18 hours and 0.16 hours before the perfusion was started. This release was not influenced by cycloheximide, sodium phenobarbitone or the injection of large amounts of unlabeled vitamin B_{12} 18 hours before the perfusion. In our studies cycloheximide and puromycin were found to be ineffective on the release of vitamin B_{12} from the labile and the stable pool. This indicates that protein synthesis is not directly involved in the release of vitamin B_{12} from the cell.

Excretion of radioactive vitamin B_{12} in the bile was low, compared with the secretion by the isolated liver cells in our study and also in comparison with the secretion by the perfused rat liver in the studies of Cooksley and Tavill (11).

In conclusion the experimental results are indicative for rapid exchange between vitamin B_{12} in the blood and a labile intracellular pool of the liver cell, presumably involving a process of adsorptive endocytosis. A small fraction of the internalized vitamin B_{12} is probably retained by the cell to meet its own metabolic requirements and to replenish the slowly releasing stable pool.

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PURIFICATION OF HUMAN TRANSCOBALAMIN II-CYANOCOBALAMIN BY AFFINITY CHROMATOGRAPHY USING A THERMOLABILE IMMOBILIZATION OF CYANOCOBALAMIN

5.1. Summary.

Transcobalamin II was isolated from Cohn fraction III of pooled human plasma with an affinity chromatography technique followed by conventional separation methods. The affinity material was prepared by a direct thermolabile attachment of hydroxocobalamin (OH-Cbl) to AH-Sepharose 4B. The specificity of the affinity ligand for Cbl-binding proteins was improved by a conversion into cyanocobalamin (CN-Cbl), with maintenance of the thermolabile linkage. The total purification procedure included successively CM-Sephadex batchwise elution, affinity chromatography, Sephacryl S 200 gel filtration and DEAE-Sepharose CL-6B ion-exchange chromatography with a linear and a concave gradient elution. The final product was obtained with a yield of 55% and a purification factor of 1.1×10^6. The specific CN-Cbl binding capacity was 0.98 mol CN-Cbl/mol TC II. In dodecyl sulphate polyacrylamide gel electrophoresis one major protein band was observed at a molecular weight of 37 000 and a faint band at a molecular weight of 29 000. A considerable shift from the first to the second band occurred, when the protein was incubated with β-mercaptoethanol or dithiothreitol before electrophoresis.

At the same time three faint protein bands appeared at molecular weights of 24 000, 18 000 and 13 000. This suggests a dissociation of the TC II molecule in smaller subunits. In polyacrylamide gel isoelectric focusing the pure preparation appeared to be composed of several isoproteins with isoelectric points ranging from pH 6.2 to 6.8. The pure material was labeled with ^{125}I using immobilized

lactoperoxidase. The labeled product was analyzed with
gel filtration and ion exchange chromatography and appeared
to be unaltered.

5.2. Introduction.

    In the preceeding chapters the binding of TC II-$\left[^{57}Co\right]$
CN-Cbl to isolated rat liver plasma membranes and the uptake
of TC II-$\left[^{57}Co\right]$ CN-Cbl by isolated rat liver parenchymal
cells have been described. Because the radioactive label was
in the vitamin moiety of the complex the results of the
experiments gave almost no data on the role and fate of the
protein part. The study of the uptake and digestion of the
transport protein necessitates the incorporation of a radio-
active label in the protein. For this the protein had to be
purified to homogeneity first. A review of the existing
methods of purification for TC II was given in section 1.2.5.
In this chapter a new procedure is presented which is based
on the method of Nexø (1,2), and which makes use of the
property of hydroxocobalamin to form a thermolabile linkage
with certain aminogroups.
    We found that the method of Nexø was inadequate for the
purification of TC II from rat or human plasma, because also
large amounts of non-TC II-plasma proteins were retained by
the affinity column, which were very difficult to remove in
later purification steps.
    A simple modification is described which effectively
reduced the non-specific binding of proteins to OH-Cbl. It
appeared to be possible to convert the immobilized OH-Cbl to
CN-Cbl by elution of the column with a buffer containing
cyanide. Under the influence of the cyanide OH-Cbl changes
to di-CN-Cbl, which after omission of KCN from the buffer
is converted to mono-CN-Cbl under maintenance of the thermo-
labile linkage. CN-Cbl has almost no affinity for plasma

proteins other than TC II and cobalophilin, in contrast to
OH-Cbl which also avidly binds to albumin and other proteins
(3,4,5,6). The effect of this modification, the result of a
representative purification procedure and the characteriza-
tion of the isolated material are presented in the following
sections.

5.3. Methods.

5.3.1. Preparation of the affinity column.

AH-Sepharose was used as the insoluble matrix. 1 g AH-
Sepharose 4B was swollen in 200 ml 0.5 M NaCl for 15 min and
washed with another 300 ml 0.5 M NaCl. The swollen Sepharose
was directly incubated with OH-Cbl in about 2 ml 0.1 M $NaHCO_3$,
0.5 M NaCl, pH 8.2. The amount of OH-Cbl, which was incubated
with the Sepharose, was about 10 times in excess of the
binding capacity of the sample from which the binding proteins
were going to be isolated. This because the average efficiency
of the coupling procedure is 20-30% and an excess of about
100% of immobilized cobalamin in comparison to the binding
capacity of the sample is thought to be desirable.

For the detection of TC II in further purification steps
AH-Sepharose was incubated with a mixture of labeled and
unlabeled OH-Cbl. $[^{57}Co]$ OH-Cbl was prepared from $[^{57}Co]$
CN-Cbl by exposure to a 60 W tungsten lamp at a distance of
15 cm for 18 hours in 8 mM HCl, according to Mahoney et al.
(7). The cuvette was thermostatically kept at $4^{\circ}C$. The
Sepharose suspension was incubated with OH-Cbl for about
18 hours at room temperature in a rotary mixer, cooled on
ice for 30 min and poured into a precooled glass column
(1.5 x 5.5 cm). The Sepharose was washed with 50 ml of 20 mM
sodium phosphate, 1 M NaCl, 10 mM KCN, buffer pH 8.2 to
convert the OH-Cbl to di-CN-Cbl. The excess of Cbl in the

effluent was measured spectrophotometrically to establish
the coupling efficiency. KCN was removed with 200 ml of the
phosphate-NaCl buffer without KCN and at the same time the
di-CN-Cbl was converted to mono-CN-Cbl. In this form the
column was used for the purification procedure.

5.3.2. Purification of human transcobalamin II.

    A representative purification procedure is described in
detail. 80 l of 10 mM sodium phosphate, 0.1 M NaCl, buffer
pH 5.2 was added to 19 kg of Cohn fraction III, and the
suspension was stirred continuously for 12 hours at 4°C.
Insoluble material was allowed to settle and the supernatant
was filtered over nylon gauze (63μ). The filtrate was stirred
for another 12 hours with 100 g of dry CM-Sephadex C-50. The
Sephadex was collected by filtration over nylon gauze and
washed first on the gauze and afterwards on a glass filter
with 10 l of 10 mM sodium phosphate, 0.05 M NaCl, buffer
pH 5.2. TC II was eluted from the Sephadex by stirring the
suspension in 1.5 l of 0.2 M Tris, 1 M NaCl, pH 8.2. The
Sephadex was washed with another 400 ml Tris buffer. The
total filtrate, about 3300 ml, was centrifuged for 2 hours
at 105 000 x g. The supernatant was passed through filter
paper (S&S 589[3]) to remove floating lipid material. The
cobalamin-binding capacity was measured and the total volume
was applied to the affinity column, which contained 210 μg
of immobilized CN-Cbl with a specific activity of 19 175
cpm/μg, at a flow rate of 100 ml/hour. The column was washed
with 400 ml cold 20 mM sodium phosphate, 1 M NaCl, 0.02%
NaN$_{3}$, pH 7.4 and during this washing the Sepharose was
resuspended and allowed to settle in order to remove
particulate material. Elution of the TC II-CN-Cbl took
place after incubation of the column for 10 hours at 37°C
and washing with about 5 ml of warm phosphate-NaCl buffer.

After another 10 hours at 37°C the remainder of the TC II-CN-Cbl and free cobalamin were eluted with 4 ml of warm buffer and the two eluates were combined.

The TC II-CN-Cbl solution was subsequently applied to a Sephacryl S 200 column (2.6 x 90 cm) and elution took place with the same phosphate buffer. The fractions containing TC II-CN-Cbl, detectable by their ^{57}Co-radio-activity, were pooled and concentrated by ultrafiltration on a YM 10 membrane (Amicon). After this concentration the buffer was changed to 50 mM Tris/HCl pH 8.25 as a preparation for the next purification step, DEAE-Sepharose ion exchange chromatography. The concentrate was put on a 0.9 x 4 cm column of DEAE-Sepharose CL-6B and eluted with a linear gradient from 0 to 225 mM NaCl in 50 mM Tris/HCl pH 8.25 with a total volume of 200 ml.

The concentrated fractions containing TC II-CN-Cbl were again brought in 50 mM Tris/HCl pH 8.25 and applied to a second DEAE-Sepharose column. For the elution a concave gradient from 0-150 mM NaCl was created by a two-pump system in which the efflux from the mixing chamber was 14 times the influx of the high salt solution into the mixing chamber (8). The total volume of the gradient was 200 ml. The fractions containing TC II-CN-Cbl were pooled and concentrated on a YM-10 ultrafiltration membrane.

The different purification products were subjected to measurements of the CN-Cbl-binding capacity or the vitamin B_{12} content and of the total protein in order to establish the specific vitamin B_{12}-binding capacity of the isolated material. The methods for the measurements of binding capacity and vitamin B_{12} concentration are described in section 2.2.2 and 2.2.3 respectively.

5.3.3. Further analytical methods.

Protein was measured according to Lowry et al. (9), using bovine serum albumin or human serum albumin protein standard (Kabi, Stockholm) as a reference. Both standards gave identical results.

Dodecyl sulphate polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (10). Electrophoresis was performed at 2.5 mA/gel until the tracking dye, pyronine Y, reached the bottom of the electrophoresis tube. The gels were fixed and stained with Coomassie brilliant blue.

Thin layer polyacrylamide gel isoelectric focusing was performed with a modification of the method of Vesterberg (11). The gel was composed of 13.5 ml 30.5% acrylamide, 15 ml 1% NN'-methylene bisacrylamide, 27 ml H_2O, 0.65 g Triton X-100, 7.5 g sucrose, 1.0 ml Ampholine pH 5-7, 1.0 ml Ampholine pH 6-8, 1 ml Ampholine 7-9, 1 ml 0.0055% riboflavin and polymerization was induced by a daylight tube lamp. The samples were applied in small(5x10x1mm) basins in the gel. The cathode solution was 0.1 M NaOH, the anode solution 0.1 M H_3PO_4.

Focusing was done in a LKB Multiphor apparatus for 90 min at $4^{o}C$. At the end of the focusing time the pH gradient was measured with an Ingold Surface electrode type 104033.104. The proteins were fixed in 50% methanol/7.5% TCA and stained with Coomassie brilliant blue.

5.3.4. ^{125}I-labeling of purified transcobalamin II-CN-Cbl.

40 mg lactoperoxidase (Boehringer, Mannheim, Germany) was dialyzed for 24 hours against 1 l of 0.1 M $NaHCO_3$ buffer pH 8.0, containing 0.5 M NaCl. The dialyzed protein was coupled to AH-Sepharose 4B according to the instructions of

the manufacturer. Unbound protein was removed by filtration
and the gel was finally washed with distilled water and
lyophilized. The total amount of lyophilized material was
520 mg.

The enzymatic activity was determined by means of a
2.2'-azino-di $\left[$ 3-ethyl-benzthiazoline sulfonate (6) $\right]$ $(NH_4)_2$
$(ABTS^R)$-coupled reaction. The increase of extinction at
436 nm was used as a measure of enzyme activity (ε_{436}^{1cm} =
29.3 $cm^2/\mu mol$).

5 mg of lyophilized lactoperoxidase - Sepharose
(LP-Sepharose) were swollen in 0.07 M potassium phosphate
buffer, pH 5.5 and added to the reaction mixture, which
contained 20 mM ABTS and about 0.25 mM H_2O_2 in the same
buffer. During the reaction the Sepharose was kept in
suspension. The enzyme activity was found to be 900 mU/mg
lyophilized LP-Sepharose and the percentage of coupled and
recovered enzyme activity was 10%.

The protein labeling was carried out according to David
(12). 0.5 mg LP-Sepharose was swollen in 0.5 ml 20 mM sodium
phosphate, 0.15 M NaCl, pH 7.4 in a 1.5 ml micro reaction
vessel. The suspension was centrifuged for 1 min at 8 000
x g and the supernatant was removed. 0.9 ml phosphate-NaCl
buffer was added to the pellet, followed by 0.1 ml (30 µg)
TC II-CN-Cbl, 0.1 ml (1 mCi) $\left[^{125}I\right]$ NaI and 0.1 ml 0.122 mM
KI. At 0, 10 and 20 min 10 µl 0.03% H_2O_2 was introduced and
the reaction vessel was rotated for 30 min. After the
incubation the mixture was centrifuged for 2' at 8 000 x g
to remove the LP-Sepharose beads and the supernatant was
collected. The Sepharose pellet was washed twice with
incubation buffer and the collected supernatants were
applied to a Sephadex G75 column (1.6 x 70 cm). The sample
was eluted with 20 mM sodium phosphate, 1 M NaCl pH 7.4. The
2 ml fractions were collected in siliconized tubes which
contained 100 µl of 1.8% human serum albumin in saline. The
latter precautions were taken in order to reduce absorption

of the labeled protein, which amounted to 62% in normal glass
tubes, 30% in siliconized glass tubes and 9% when also
albumin was added.

The TC II-containing fractions were pooled and concen-
trated by ultrafiltration. The vitamin B_{12} content was
measured by radioassay and a specific ^{125}I-radioactivity of
190 cpm/pg CN-Cbl was calculated.

5.4. Results.

5.4.1. Preliminary observations.

Our improvement of the affinity chromatography technique
was based on the results of purifications of transcobalamin
II from rat plasma using the unmodified method of Nexø (4).
When rat plasma with a total CN-Cbl-binding capacity of
0.16 nmole was applied to a OH-Cbl-Sepharose column, 59
nmoles of protein-bound OH-Cbl were recovered in the 37°C
eluate. This means that other plasma proteins also showed
a high affinity for the OH-Cbl in the column. Because it is
known that for instance serum albumin readily binds OH-Cbl
but not CN-Cbl (13) it was tried to prepare a CN-Cbl-
Sepharose column. CN-Cbl-Sepharose was obtained from OH-Cbl-
Sepharose by successive rinses with a CN^- containing and a
CN^--free buffer so that OH-Cbl was converted first to di-CN-
Cbl and then to mono-CN-Cbl, as was illustrated by the
absorption spectra of the 37°C eluates in each of the three
stages of preparation (figure 5.1). When the rat serum was
applied to such a CN-Cbl-Sepharose column all protein-bound
CN-Cbl in the eluate was bound to TC II, which means that
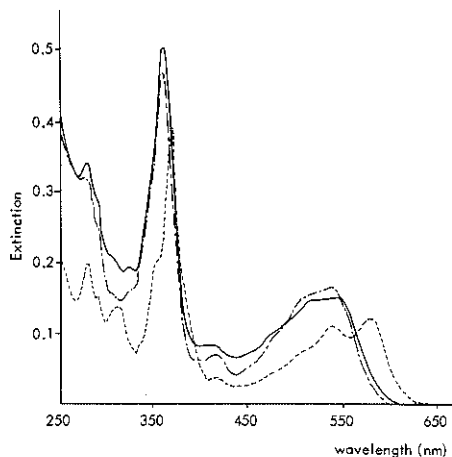the non-specific binding was completely eliminated.

fig. 5.1. Spectra of affinity column eluates. —·—·—·, hydroxo-
cobalamin from an unconverted column; — — — — —,
dicyanocobalamin from a converted column with CN^-
in the elution buffer; ————, cyanocobalamin from
a converted column which was previously rinsed
with CN^- free buffer. All samples were diluted in
the respective elution buffers and the same buffer
was used as a reference. The spectra were recorded
on a Perkin Elmer 124 spectrophotometer.

5.4.2. Purification of human transcobalamin II.

With this improved affinity chromatography technique the
purification of TC II was started with Cohn fraction III from
human plasma. The total vitamin B_{12}-binding capacity of the
Cohn fraction solution was 170 µg. The insoluble material
contained also some binding capacity, but a second extraction
was not worthwhile, because the yield and the specific binding
activity were low. The vitamin B_{12}-binding capacity of the
first supernatant was used as the 100% value for the
calculation of the yield of the purification.

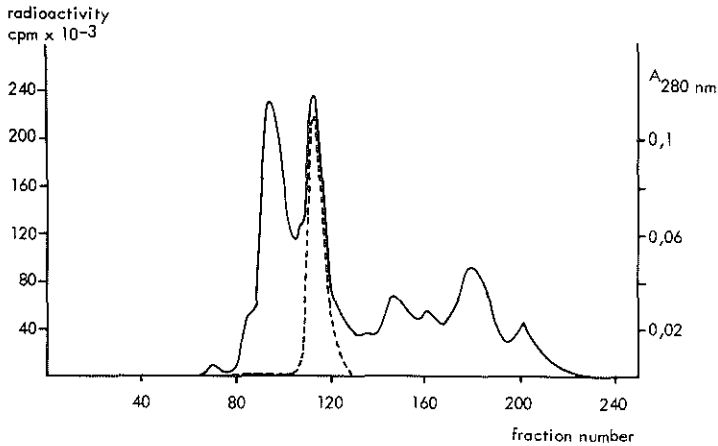Using batchwise CM-Sephadex elution the TC II was

fig. 5.2. Elution profile of the affinity column eluate on a
Sephacryl S 200 column (2.6 x 90 cm) equilibrated
with 20 mM sodium phosphate, 1 M NaCl buffer, pH
7.2. ————, A_{280}; -------, ^{57}Co-radioactivity.
Fraction volume was 2.8 ml and the elution
velocity was 10 ml/hour.

recovered from the supernatant in a volume of 3.3 l, with a
17-fold purification and only a 10% loss of binding capacity.
     The specific vitamin B_{12}-binding capacity of the eluate
from the affinity column could not be calculated directly,
because the eluate contains a mixture of free and bound Cbl.
The amount of bound cyanocobalamin was determined in the
following purification step: the Sephacryl S 200 column. The
total amount of bound cyanocobalamin was divided by the total
amount of protein in the eluate of the affinity column to
calculate the specific binding capacity. The elution profile
of the Sephacryl S 200 column is given in figure 5.2. The
specific binding capacity was 16.3 µg vitamin B_{12}/mg protein,
which suggests a purity of about 50%. Further purification
was carried out with DEAE-Sepharose CL-6B ion exchange
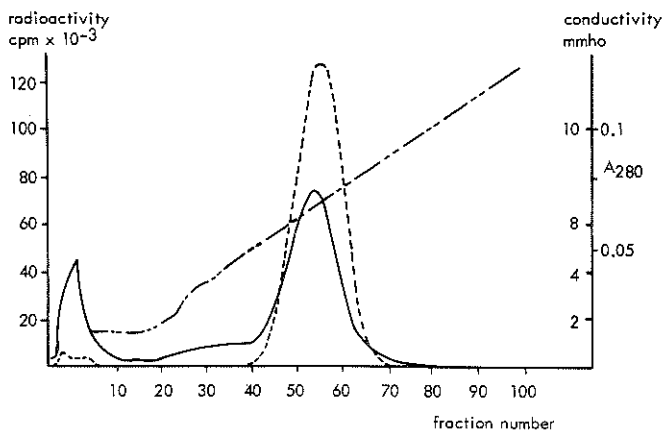chromatography and the elution profile with a linear salt

fig. 5.3. Elution pattern of the first DEAE-sepharose CL-6B column. The column (0.9 x 4 cm) was equilibrated with 50 mM Tris/HCl pH 8.25 and elution took place with a linear salt gradient from 0 to 225 mM NaCl with a volume of 200 ml.——— , A_{280}; --------
^{57}Co-radioactivity; —··—··— , conductivity. Fraction volume was 2 ml and the elution velocity was 15.8 ml/hour.

gradient is presented in figure 5.3. The shoulder of non-TC II protein before the TC II peak was eliminated by DEAE-Sepharose chromatography with a concave salt gradient (figure 5.4). Measurement of protein and vitamin B_{12} in the final product gave a specific binding activity of 34.8 µg/mg or 0.98 mole CN-Cbl per mole TC II (molecular weight TC II 38 000), which means that the preparation was almost pure. The purification factor was 1.1×10^6 with a yield of 55%. A summary of the results of the purification procedure is given in table 5.1.

Table 5.1.   *Purification of human transcobalamin II-cyanocobalamin*

| step of purification	volume (ml)	total protein (mg)	total CN-Cbl-binding capac. (µg)	specific CN-Cbl-binding (μg/mg)	capacity (mol/mol)	purification factor	yield (%)
Cohn fraction III (solubilized)	95000	5.36×10^6	170	3.17×10^{-5}	8.9×10^{-7}	1	100
CM-Sephadex batchwise elution	3300	0.27×10^6	153	5.66×10^{-7}	0.16×10^{-4}	18	90
affinity chromatography on CN-Cbl-Sepharose	11.4	31	120	3.87	0.108	1.22×10^5	71
Sephacryl S200 gel filtration	7.1	5.84	95.2	16.3	0.46	5.14×10^5	56
DEAE-Sepharose CL-6B linear gradient	6.9	3.93	91.0	23.2	0.65	7.32×10^5	54
DEAE-Sepharose CL-6B concave gradient	9.0	2.67	93.0	34.8	0.98	1.10×10^6	55
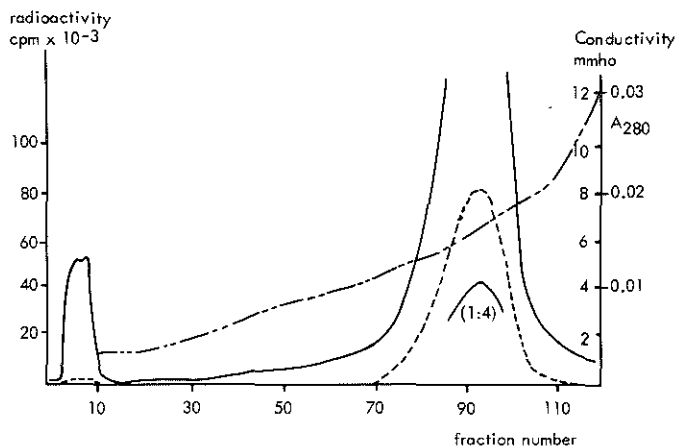
fig. 5.4. Elution pattern of the second DEAE-sepharose CL-6B
column. The column (0.9 x 4 cm) was equilibrated
with 50 mM Tris/HCl pH 8.25 and elution took place
with a concave gradient from 0 to 150 mM NaCl with
a total volume of 200 ml. ————— , A_{280}; ---------,
^{57}Co-radioactivity; —··—··—, conductivity. Fraction
volume was 2.0 ml and the elution velocity 15.2
ml/hour.

5.4.3. Characterization of the purified transcobalamin II.

The pure transcobalamin II-CN-Cbl was subjected to
dodecyl sulphate polyacrylamide gel electrophoresis under
reducing and non-reducing conditions (figure 5.5). In electro-
phoresis without a reducing agent a single major band was
observed at a molecular weight of 37 000 and a faint band,
less than 5% in densitometric analysis, with a molecular
weight of about 29 000, even if as much as 75 μg TC II was
applied. The incubation with 1% β-mercaptoethanol, 10 mM or
50 mM dithiothreitol resulted in a 50% decrease of 37 000
molecular weight material, an increase of the 29 000 band,
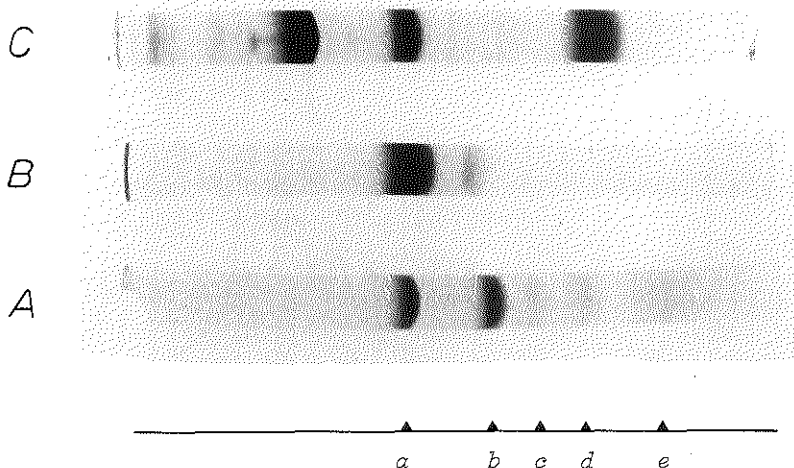and the appearance of some faint bands at 24 000, 18 000

fig. 5.5. *Dodecyl sulphate polyacrylamide gel electrophoresis*
of 12.5 μg of transcobalamin II (A and B) and of a
mixture of albumin, aldolase and myoglobin (C, 10
μg each). Preparation and electrophoresis of the
samples in A and C were carried out in the presence
of 50 mM dithiotreitol. The characters a,b,c,d, and
e refer to fig. 5.6 in which the relative mobilities
are used for the calculation of the molecular weights.

and 13 000. The molecular weights were estimated by comparison
with three marker proteins (figure 5.6). From these data it
is concluded that about 50% of the TC II dissociates into
smaller subunits under reducing conditions. Electrophoresis
patterns were not different whether preincubation was carried
out for 2 hours at 37°C or for 3 min at 100°C.

Polyacrylamide gel isoelectric focusing of the pure
TC II-CN-Cbl was initially not successful, because the
protein precipitated during the procedure even before it had
reached the area of its isoelectric point. This was probably
a result of the poor solubility of TC II at low ionic strength.
The addition of 1% Triton X-100 improved the solubility.
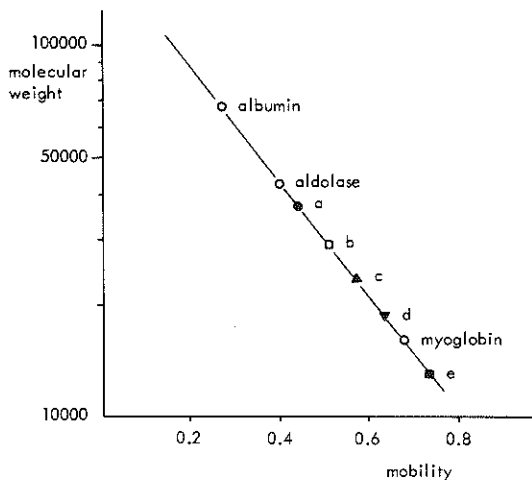Figure 5.7 shows an IEF-pattern of the pure TC II preparation

fig. 5.6. Plot of the molecular weights of the three marker proteins versus their relative mobilities on dodecyl sulphate polyacrylamide gel electrophoresis (figure 5.5). The characters a,b,c,d and e represent the different polypeptide chains in the TC II preparations: a, 37 000; b, 29 000; c, 24 000; d, 18 000; e, 13 000 molecular weight.

in a pH gradient from 4.9 to 7.4. The protein is concentrated in an area from pH 6.2 to pH 6.8, in which at least two double bands around pH 6.30 and 6.45 are visible.

5.4.4. ^{125}I-labeling of the isolated transcobalamin II-cyanocobalamin.

Portions of 30 µg pure TC II were labeled with ^{125}I with the use of immobilized lactoperoxidase. After the incubation the labeled protein and the other constituents were separated by gel filtration on Sephadex G-75. A representative elution pattern is shown in figure 5.8.

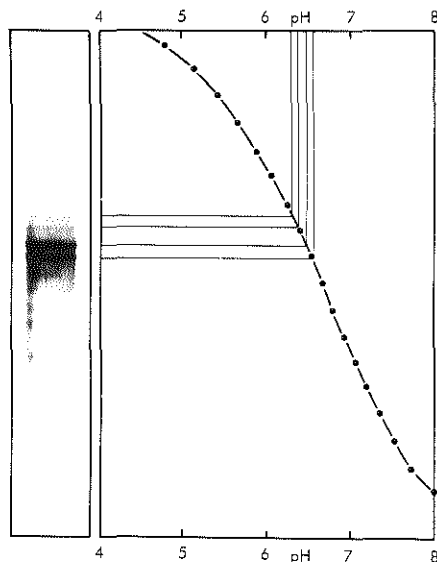The symmetrical radioactivity peak completely coincided

fig. 5.7. Polyacrylamide gel isoelectric focusing of 45 µg
of pure TC II. The diagram indicates the course of
the pH over the gradient from anode to cathode.

with the light absorption peak at 280 nm around fraction
32 and the elution volume was the same as for TC II-$[^{57}Co]$
CN-Cbl. This indicates that the molecular weight did not
change by the labeling procedure.

A further control of the integrity of the labeled
protein was performed by means of ion exchange chromatography.
A small amount of $[^{125}I]$ TC II-CN-Cbl and about the same
amount of partially purified TC II-$[^{57}Co]$ CN-Cbl were mixed
and separated on a DEAE-Sepharose column with a linear salt
gradient. The elution pattern is shown in figure 5.9. Both
peaks of radioactivity were eluted at exactly the same salt
concentration, which indicates that the labeling procedure
did not cause a significant change of the isoelectric point.
The most important characteristic of the labeled protein,
its function in the cellular uptake of vitamin B$_{12}$, is
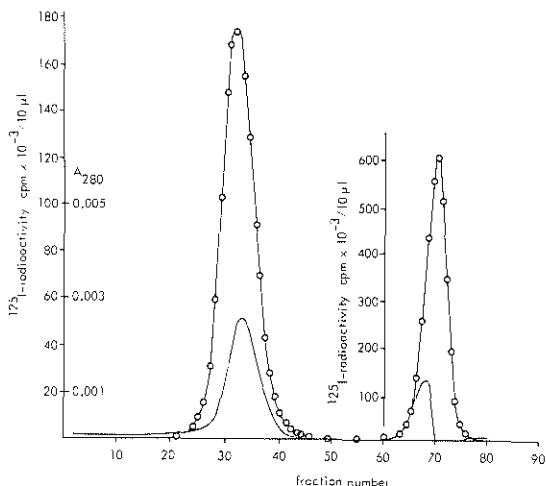analysed in the following chapter.

fig. 5.8. Separation of ^{125}I-labeled TC II-CN-Cbl and free
$\left[^{125}I\right]$ NaI by gel filtration on a Sephadex G 75
column (1.6x65 cm). Elution buffer was 20 mM sodium
phosphate, 1 M NaCl, pH 7.4. o————o ^{125}I-radioacti-
vity; ———— A_{280}. Fraction volume was 1.8 ml and
the elution velocity was 25 ml/h.

5.5. Discussion.

      Purification of transcobalamin II with conventional
methods is unsatisfactory, because it is almost impossible
to obtain a fair yield of the pure product. The affinity
chromatography technique described by Allen and Majerus (13),
gave better recovery and has made the characterization of
the purified material possible. This method has the
disadvantage that it requires strong protein denaturation
for a rather long time, which may inactivate the trans-
cobalamin. Weiss et al. (14) e.g. have reported that human
intrinsic factor, which was purified by the method of
Allen et al. (13) had a decreased affinity for the intestinal
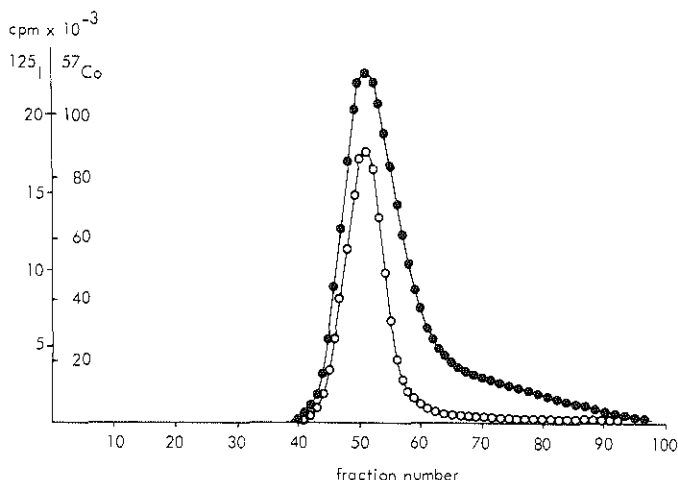receptor, and denaturation of cobalophilin with this method

Fig. 5.9. Elution profile of a mixture of equal amounts of
         partially purified TC II-$[^{57}Co]$ CN-Cbl and $[^{125}I]$
         TC II-CN-Cbl on DEAE-Sepharose CL-6B (bed volume
         2.5 ml). Elution was carried out with a linear
         gradient from 50 mM Tris, pH 8.25 to 50 mM Tris,
         225 mM NaCl, pH 8.25. Fraction volume was 2 ml
         and the elution velocity was 16 ml/h. ●────●
         ^{57}Co-radioactivity; o────o ^{125}I-radioactivity.

was suggested by Stenman (15).

     Nexø (1) has described an easier and more gentle
affinity chromatography method for the purification of
intrinsic factor and cobalophilins, in which OH-cobalamin
is attached to immobilized 3.3'-diaminodipropylamine by a
thermolabile linkage. When unsaturated serum is applied to
such a column, only the vitamin B_{12}-binding proteins are
retained and eluted as saturated binding proteins from the
column at 37°C. Initially we tried this method for the
isolation of transcobalamin II from rat plasma. However, in
the eluate of the affinity column only a very small fraction
of the protein-bound OH-Cbl was coupled to TC II. Therefore,
we investigated the possibility to incorporate CN-Cbl, which

shows less non-specific binding, in the affinity column.

Direct incorporation proved to be impossible because CN-Cbl has no affinity for the 3.3'-diaminodipropylamine molecule. However, we observed that the OH-Cbl, when attached to the insoluble matrix, could be converted to CN-Cbl, by rinsing with a buffer containing cyanide, without losing the thermolabile linkage. Non-specific binding was completely absent because no other vitamin B_{12}-binding proteins than TC II were detectable in the 37°C eluate.

A further simplification of the method of Nexø was the coupling of OH-Cbl directly to AH-Sepharose instead of to the 3.3'-diaminodipropylamine-substituted CN-Br-Sepharose.

Human transcobalamin II was purified from Cohn fraction III to homogeneity by CM-Sephadex ion exchange chromatography, affinity chromatography, gel filtration and DEAE ion exchange chromatography, successively. The use of isoelectric precipitation for the final purification, reported by Savage et al. (16), appeared to be irreproducible. Excellent separation of TC II from contaminating proteins was obtained with the Sephacryl S-200 column. Another important improvement in the yield of the overall procedure was the use of Amicon YM-10 ultrafiltration membrane for which TC II has only very low affinity in contrast to UM or PM-type membranes.

The final product was virtually pure on the basis of the specific binding activity, calculated to be 0.98 mol CN-Cbl/ mol TC II. A slightly lower specific binding activity, 0.87 mol CN-Cbl/mol TC II was obtained when the amount of CN-Cbl in the final preparation was calculated on the basis of the specific radioactivity of the OH-Cbl solution, which was used for the preparation of the affinity column. A possible explanation for this small difference is, that the $\left[^{57}Co\right]$ OH-Cbl, as a result of the conversion with light in an acid environment, contained derivatives, which adhered to the AH-Sepharose but had lost their binding affinity for the TC II molecule.

The purity of the material was further established
by means of dodecyl sulphate polyacrylamide gel electro-
phoresis. The small protein band at a molecular weight of
29 000 does not seem to be a contamination but rather a sub-
fraction of TC II, because this band intensified considerably
when preincubation and electrophoresis were carried out in
the presence of reducing agents. In addition components with
molecular weights of 24 000, 18 000 and 13 000 appeared. This
suggests that our preparation contained a mixture of TC II
as a single polypeptide chain with a molecular weight of
37 000, and of TC II composed of two separate polypeptide
chains with molecular weights of either 29 000 and 13 000
or 24 000 and 18 000. Similar observations were described
by Allen et al. (13,17), who concluded that native TC II is
a single polypeptide chain and that the smaller polypeptide
chains had been formed by an internal cleavage. We have tried
to determine, whether proteolysis during the purification had
caused the formation of the smaller fragments. However, the
electrophoresis of a TC II-preparation, which was purified
in the presence of diisopropylfluorophosphate in order to
prevent proteolysis, gave exactly the same results. Therefore,
whenever the formation of the smaller polypeptide chains
was due to proteolysis this must have taken place before the
purification.

Polyacrylamide gel isoelectric focusing of pure TC II is
extremely difficult because of the strong tendency of TC II
to precipitate at low ionic strength. Recently Marcoulis et
al. reported that they were unable to establish a typical
electrofocusing pattern in a sucrose gradient (18). In our
experience the solubility in the gel could be improved best
by the addition of Triton X-100. TC II concentrated in a
rather broad area between pH 6.2 and 6.8 in at least four
discernable protein bands. These protein bands may represent
TC II molecules in the different polypeptide chain
configurations, which were demonstrated in the dodecyl

sulphate polyacrylamide gel electrophoresis, but another
explanation is the occurrence of isoprotein forms. Genetic
polymorphism of transcobalamin II has been reported by
Frater-Schröder et al. (19) and by Daiger (20). Because
there is evidence that TC II is synthesized by different
tissues (21,22) it is tempting to speculate that these
tissues produce slightly different TC II molecules.

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BINDING, UPTAKE AND DEGRADATION OF $[125I]$ TRANSCOBALAMIN II-CYANOCOBALAMIN
BY ISOLATED RAT LIVER CELLS AND THE PERFUSED RAT LIVER

6.1. Summary.

Isolated rat liver parenchymal cells were incubated
with human TC II-CN-Cbl, which had been purified to
homogeneity by means of affinity chromatography and
enzymatically labeled with radio-iodine. The binding and
uptake followed the same biphasic pattern as the binding and
uptake of TC II-$[^{57}Co]$ CN-Cbl, i.e. a steep rise of cell
bound radioactivity in the first few minutes, and a continuous
temperature dependent increase in the following two hours.
These steps are interpreted as a rapid binding of the complex
to the outer cell membrane and a gradual entrance of the
complex into the cell. With a lag of about 15 min acid-
soluble radioactive material appeared in the medium as a
result of intracellular proteolysis of TC II and increased
linearly during the next two hours of incubation to about
1.5 times the amount of radioactive TC II inside the cell.
The rate of uptake, i.e. the increase with time of intra-
cellular radioactivity plus degradation of TC II, as a
function of the extracellular concentration of TC II-CN-Cbl
showed a partial saturability. Addition of 10 nM unlabeled
pure TC II-CN-Cbl led to a suppression of the saturable
component of the uptake. After correction of the total
uptake for these non-specific components, a curve of specific
saturable uptake was obtained. The uptake of human $[^{125}I]$
TC II-CN-Cbl was suppressed by purified rat TC II-CN-Cbl
indicating that human and rat TC II compete for the same
binding sites on the cell surface. Binding and uptake of
human $[^{125}I]$ TC II-CN-Cbl were not affected by EDTA or EGTA.
The lysosomotropic agent chloroquine reduced the degradation

of TC II and consequently caused an accumulation of radio-
active TC II within the cell. In additional studies using
liver perfusion the uptake and degradation of TC II were
quantitatively and qualitatively similar to the uptake and
degradation by liver cells incubated in suspension.

6.2. Introduction.

    The results described in chapter 4 suggested that TC II-
vitamin B_{12} is taken up by the liver cell by a pinocytotic
mechanism and subsequent inclusion in the lysosomal system.
Especially the experiments with the lysosomotropic agent
chloroquine made it likely that proteolysis of TC II is a
prerequisite for the normal intracellular transport of
vitamin B_{12}. In this chapter experiments are described which
were designed to find additional evidence for this proteolytic
process. For this purpose isolated liver cells and the
perfused rat liver were incubated with ^{125}I-labeled TC II and
the binding, uptake and degradation of TC II degradation
products have been measured.

6.3. Methods.

6.3.1. Preparation of the liver cell suspension.

    Liver parenchymal cells were isolated as described in
section 2.4. The cells were washed and finally suspended in
DMEM in a concentration of 3.6 x 10^6/ml. All glassware was
siliconized.

6.3.2. Preparation of human $[^{125}I]$ TC II-CN-Cbl and rat TC II-CN-Cbl.

Purification and ^{125}I-labeling of human TC II-CN-Cbl
have been described in chapter 5. It was established that
the ^{125}I-labeled product did not differ from partially
purified TC II-$[^{57}Co]$ CN-Cbl in gel filtration and ion
exchange chromatography.

Rat TC II was partially purified by means of affinity
chromatography. 300 ml rat serum was centrifuged for 1 hr at
105 000 x g and applied to a CN-Cbl-Sepharose column, which
was prepared by incubation of 1 g AH-Sepharose 4B with 5 μg
OH-Cbl and converted to CN-Cbl-Sepharose as described in
section 5.3.1. TC II was eluted at 37°C in 15 ml 20 mM sodium
phosphate, 1 M NaCl, pH 7.4 and bovine serum albumin was
added to a concentration of 4 mg/ml. The total solution was
dialysed for 72 hours against 3 x 1 l DMEM at 4°C,
centrifuged for 1 hour at 105 000 x g and the CN-Cbl
concentration in the supernatant was determined by radio-
assay. The purification factor was estimated to be about
6 x 10^4, analogous to the affinity chromatography step in
the purification of human TC II. The yield of TC II-CN-Cbl
in the final product was 96%.

6.3.3. Standard incubation procedure.

The standard incubation was identical with the procedure
described in section 4.3.3 with the exception that in the
present experiments $[^{125}I]$ TC II-CN-Cbl was used.

Degradation of TC II was judged by the appearance of
acid soluble material in the medium and the cells. During
the incubation of $[^{125}I]$ TC II-CN-Cbl with the cells,
duplicate 0.5 ml samples were taken and centrifuged through
silicon oil as described (section 2.5). 0.2 ml aliquots of

the supernatant were mixed with 0.3 ml 3.3% bovine serum
albumin and 0.5 ml 20% perchloric acid. After incubation for
30 min at 4°C the precipitate was removed by centrifugation
for 2 min at 8 000 x g and 0.8 ml supernatant was removed
and counted. Blank values were obtained by incubations of
$\left[^{125}I\right]$ TC II-CN-Cbl without cells under the same conditions.
Degradation of TC II was defined as the difference between
the amount of acid soluble radioactivity at any time of
incubation and the corresponding blank value. The intra-
cellular degradation products were determined by measuring
the radioactivity in 0.05 ml aliquots of the perchloric
acid layer of the silicon oil centrifugation tubes. In
general these values were low and negligible.

6.3.4. Liver perfusion.

    Perfusion of rat livers was performed in situ at 37°C
under initial ether anesthesia. The cannulation of the portal
vene and the inferior vena cava was carried out as described
in 2.4. Before insertion of the perfusion cannulas the main
bile duct was cannulated with a Braunule OG20. The liver was
first perfused with about 200 ml DMEM with 0.09% human serum
albumin to wash out the blood and then the perfusate was
changed to DMEM with 0.09% human serum albumin and 450 pM
$\left[^{125}I\right]$ TC II-CN-Cbl. The recirculating extracorporeal
perfusion volume was 100 ml,from which 2 ml samples were
taken at 5, 15, 30, 60, 90 and 120 min for determination of
the total amount of radioactivity and the amount of acid
soluble ^{125}I-radioactivity. Biliary excretion of radioactivity
was determined in bile samples, collected over 30 min periods.
At the end of the perfusion the liver was rinsed with fresh
TC II-CN-Cbl free perfusate, excised and the total radio-
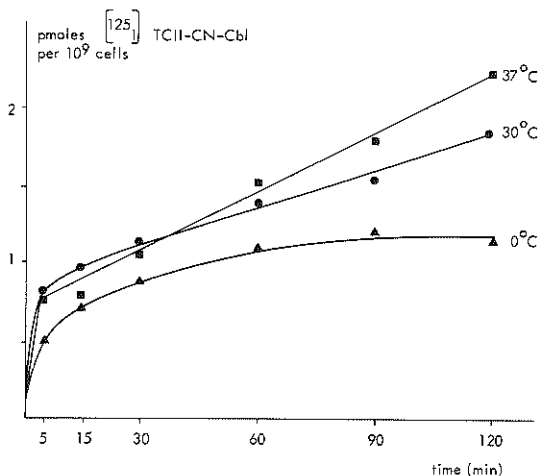activity in the liver was measured.

fig. 6.1. Binding and uptake of $\left[^{125}I\right]$ TC II-CN-Cbl by isolated liver parenchymal cells at three different temperatures. The concentration of $\left[^{125}I\right]$ TC II-CN-Cbl was 320 pM. The points are the mean of duplicate measurements.

6.4. Results.

6.4.1. Binding, uptake and degradation by isolated liver cells.

The studies on binding and uptake of $\left[^{125}I\right]$ TC II-CN-Cbl by isolated rat liver parenchymal cells showed a rapid binding to the surface of the cells in the first few minutes of the incubation, and a gradual temperature dependent uptake in the following two hours (figure 6.1). The distinction between binding and uptake was made on the basis of the biphasic nature of the time dependent uptake as described in section 4.4.1. At 0°C the initial increase in the cell-associated radioactivity was less rapid and the binding seemed to go to completion in the next 60 min.

The production of acid soluble material at the three different temperatures, illustrated in figure 6.2, started
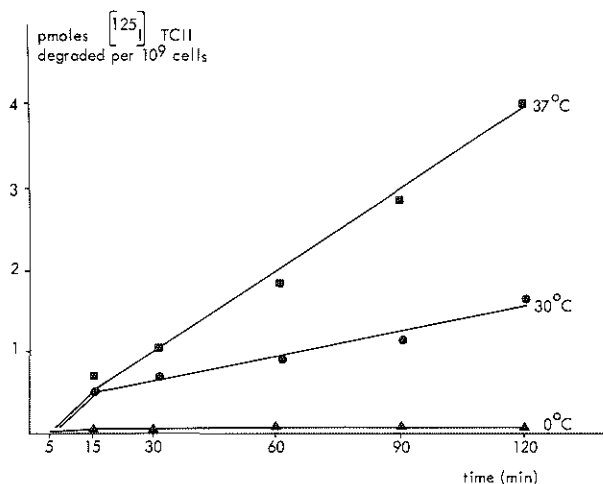
fig. 6.2. Degradation of $[^{125}I]$ TC II-CN-Cbl by isolated
liver parenchymal cells at three different
temperatures. The concentration of $[^{125}I]$ TC II-
CN-Cbl was 320 pM. The points are the mean of
duplicate measurements.

between 5 and 15 min of incubation and continued in a linear
fashion for the next two hours, at least at 30°C and 37°C.
At 0°C no production of acid soluble material was observed.
From this experiment it can be calculated that at 37°C about
75% of the TC II taken up by the cells is degraded during
the two hours of incubation and secreted as acid soluble
material.

$[^{125}I]$ TC II-CN-Cbl incubated in medium without cells
remained completely intact as is demonstrated in figure 6.3.
By incubation with a cell homogenate, which was obtained by
sonication of an amount of cells equal to the standard
incubation, no acid soluble material was formed at the normal
pH 7.4, whereas at pH 5.6 TC II was degraded rapidly. With
the cell homogenate free lysosomal proteolytic enzymes are
introduced in the incubation mixture, but these enzymes have
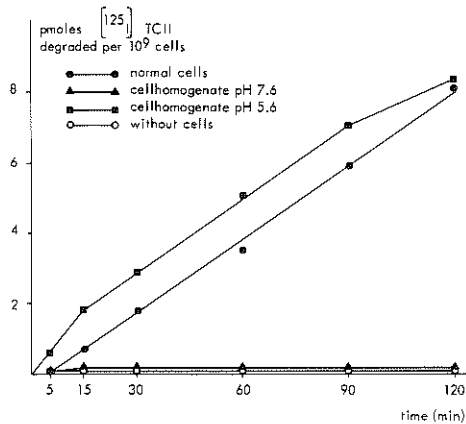an acid pH optimum and are inactive at pH 7.4. Therefore the

fig. 6.3. Degradation of $[^{125}I]$ TC II under various
circumstances measured by the appearance of acid
soluble material in duplicate samples of the medium.
The $[^{125}I]$ TC II-CN-Cbl concentration was 730 pM.

absence of acid soluble material in the incubations without
cells or with disrupted cells at pH 7.4 indicates that in
the normal incubations no other factors than the intact
viable cells are responsible for the observed degradation.

6.4.2. Uptake and degradation by the perfused liver.

The uptake and degradation of $[^{125}I]$ TC II-CN-Cbl by the
perfused liver are summarized in figure 6.4. Perfusions were
carried out with 10 mM NH_4Cl, with 2.5 mM iodoacetamide or
without special additives. NH_4Cl has an inhibiting effect
on lysosomal proteolysis by way of an increase in the intra-
lysosomal pH (1), whereas iodoacetamide causes a non-selec-
tive, irreversible inactivation of enzymes with essential
SH-groups (2). The production of acid soluble material was
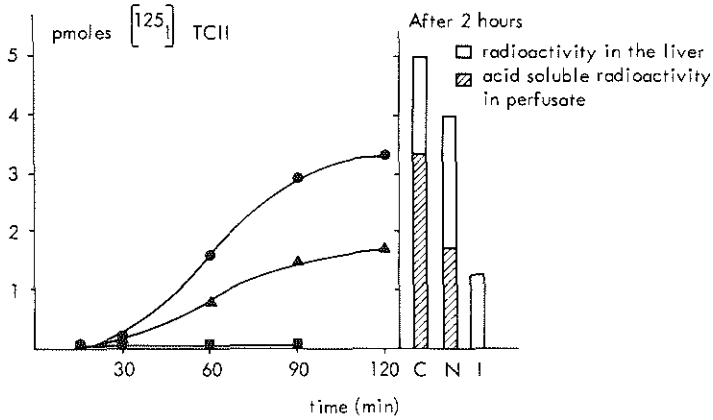measured with 30 min intervals and the liver radioactivity

fig. 6.4. *Degradation and uptake of $\left[^{125}I\right]$ TC II-CN-Cbl by perfused rat livers. On the left side the increase in acid soluble material in the perfusate is plotted versus the time of perfusion:* ●━━━●*, control;* ▲━━━▲*, with 10 mM NH_4Cl;* ■━━━■*, with 2.5 mM jodoacetamide. On the right the distribution of radioactivity in the liver and the acid soluble radioactivity in the perfusate at 120 min is illustrated. C: control cells, N: NH_4Cl and I: iodoacetamide.*

was measured after 120 min of perfusion.

In the control experiment the degradation was about twice the amount of radioactivity in the liver at 120 min. NH_4Cl caused a decrease in the production of acid soluble material and an accumulation of intracellular radioactivity, but the total amount of processed TC II was 20% less.

In the presence of iodoacetamide no degradation occurred within 90 min, after which the perfusion had to be discontinued, because of extensive leakage of the liver tissue. There was little difference in the amount of liver radioactivity in comparison with the control experiment.

Assuming that the total liver contains about 9×10^8 parenchymal cells the uptake in the perfusion experiment was about 1.8 pmoles/10^9 cells, whereas the degradation was about 3.6 pmoles/10^9 cells. In comparison with similar data obtained with isolated liver cells (6.4 pmoles/10^9 cells for binding, uptake and degradation at 400 pM $[^{125}I]$ TC II-CN-Cbl) the values are of the same order of magnitude.

The excretion of ^{125}I-activity in the bile was low, 0.1 pmole in the control experiment, 0.05 pmole in the presence of NH_4Cl and 0.005 pmole with iodoacetamide. With iodoacetamide the bile production stopped after 60 min.

6.4.3. Binding, uptake and degradation in relation to the
       concentration of TC II-vitamin B_{12}.

The competitive effect of unlabeled partially purified rat TC II-CN-Cbl on $[^{125}I]$ TC II-CN-Cbl at a concentration of 420 pM was studied (figure 6.5) and it was demonstrated that with 5 nM unlabeled rat TC II-CN-Cbl $[^{125}I]$ TC II-CN-Cbl binding was depressed to zero, the uptake to 45% and the degradation to 67% of the original level. This suggests either a relatively high level of non-specific uptake of $[^{125}I]$ TC II at this $[^{125}I]$ TC II-CN-Cbl concentration or a partial competition between human $[^{125}I]$ TC II-CN-Cbl and rat TC II-CN-Cbl.

To discriminate between these two possibilities a second experiment was carried out in which the competition between human $[^{125}I]$ TC II-CN-Cbl and unlabeled human TC II-CN-Cbl was investigated. The unlabeled TC II was purified by affinity chromatography on CN-Cbl-Sepharose, as described in chapter 5, starting with the CM-Sephadex eluate from Cohn fraction III. In this experiment several concentrations of $[^{125}I]$ TC II-CN-Cbl in the range from 100-700 pM were added to the liver cells with and without 10 nM unlabeled TC II-CN-
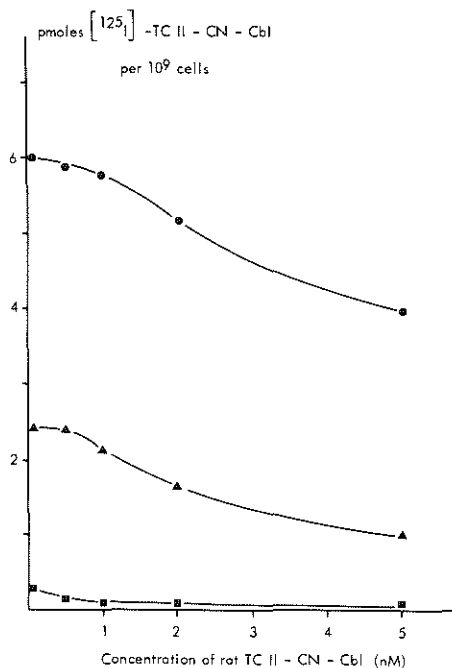
fig. 6.5. The competitive effect of unlabeled rat TC II-CN-
           Cbl on the binding, uptake and degradation of
           human $[^{125}I]$ TC II-CN-Cbl. The concentration of
           $[^{125}I]$ TC II-CN-Cbl was 420 pM. ■————■, binding to
           the cell surface; ▲————▲, total cell-bound $[^{125}I]$
           TC II at 120 min and ●————●, total amount of acid
           soluble ^{125}I-radioactivity at 120 min.

Cbl, similar to the experiments in section 4.4.1.

     Figures 6.6 and 6.7 show binding and uptake plus
degradation of $[^{125}I]$TC II-CN-Cbl in the two different series
of incubations at four concentrations of $[^{125}I]$ TC II. The
rate of uptake plus degradation was calculated from the slope
of the linear part of the curves. When these rates were
plotted against the concentration of $[^{125}I]$ TC II-CN-Cbl the
rate of uptake plus degradation in the absence of unlabeled
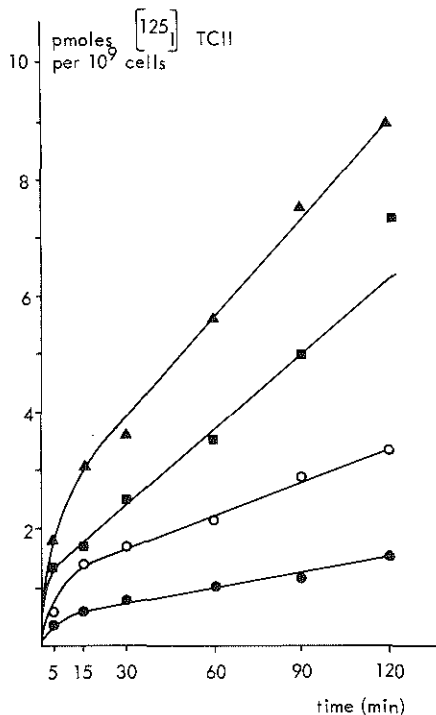human TC II appeared to be partially saturable (fig. 6.8).

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fig. 6.6. *Effect of concentration of $\left[^{125}I\right]$ TC II-CN-Cbl on the binding and uptake plus degradation of $\left[^{125}I\right]$ TC II by liver cells;* ●————●,*95 pM;* ○———— ○,*200 pM;* ■————■,*400 pM and* ▲————▲,*600 pM.*

The uptake plus degradation in the presence of unlabeled human TC II was not saturable and increased linearly with time. The conclusion from these data is that the observed uptake plus degradation is the sum of a specific and a non-specific process. The non-specific component is measured in the presence of unlabeled TC II and the specific component is calculated by subtraction of the non-specific from the total uptake plus degradation. The specific uptake plus degradation became saturated at a level of 1.2 pmole/ 10^9 cells at a concentration of about 500 pM $\left[^{125}I\right]$ TC II-CN-Cbl. Half-maximal uptake plus degradation occurred at a
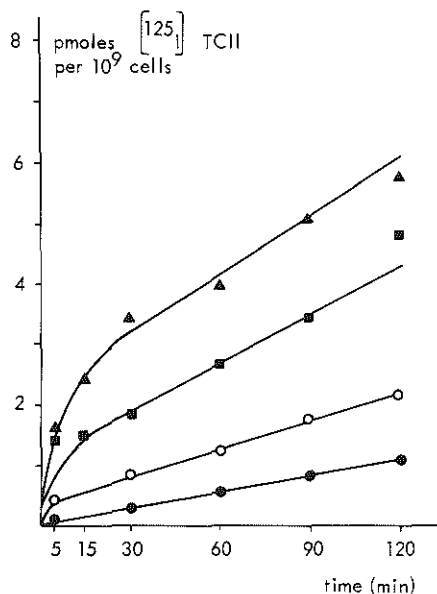
117

fig. 6.7. *Effect of the concentration of* $\left[^{125}I\right]$ *TC II-CN-Cbl*
on the binding and uptake plus degradation of
$\left[^{125}I\right]$ *TC II by liver cells in the presence of*
10 nM pure human TC II-CN-Cbl. ●————●,*95 pM;* ○————○,
200 pM; ■————■,*400 pM and* ▲————▲,*600 pM.*

concentration of 220 pM. When the kinetics of the binding of
$\left[^{125}I\right]$ TC II-CN-Cbl were analysed a similar procedure was
followed. The binding in the absence of unlabeled TC II-CN-
Cbl seemed to be partially saturable and the binding in the
presence of TC II-CN-Cbl was a linear function of the
concentration. By subtraction of the binding in the presence
of unlabeled TC II a curve of saturable specific binding was
obtained (figure 6.9). Half-maximal binding was reached at
a concentration of about 130 pM.

When the competitive effect of 10 nM human TC II at 400
pM $\left[^{125}I\right]$ TC II-CN-Cbl is compared with the competitive effect
of 5 nM rat TC II-CN-Cbl (figure 6.6), the conclusion seems
to be justified that there is no essential difference between
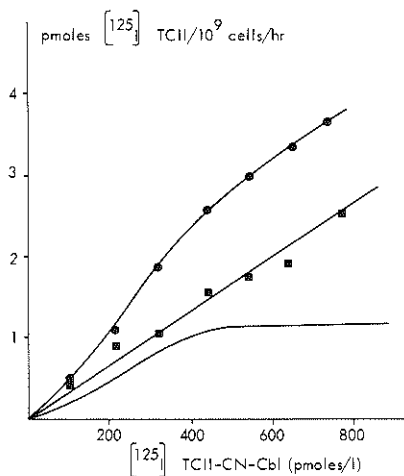human and rat TC II with regard to the affinity for the

fig. 6.8. The rate of uptake plus degradation of $[^{125}I]$
TC II-CN-Cbl, represented by the slope of the
linear part of the curves in the figures 6.6 and
6.7, as a function of the concentration of $[^{125}I]$
TC II-CN-Cbl. ●———●,rate of uptake plus
degradation measured in the absence of unlabeled
TC II-CN-Cbl; ■———■,rate of uptake plus
degradation in the presence of 10 nM unlabeled
TC II-CN-Cbl; ——— ,curve of specific uptake plus
degradation in dependence of the concentration of
$[^{125}I]$ TC II-CN-Cbl, obtained by subtraction of the
second curve from the first (top) curve.

specific binding sites.

6.4.4. Effects of agents on binding, uptake and degradation.

EDTA and EGTA had no effect on the binding, uptake and
degradation of $[^{125}I]$TC II, when studied at about 100 and
600 pM $[^{125}I]$TC II-CN-Cbl. Both chelators were present in
a concentration which was 1 mM in excess of the bivalent cat-
ions. Colchicine, which is an inhibitor of microtubular
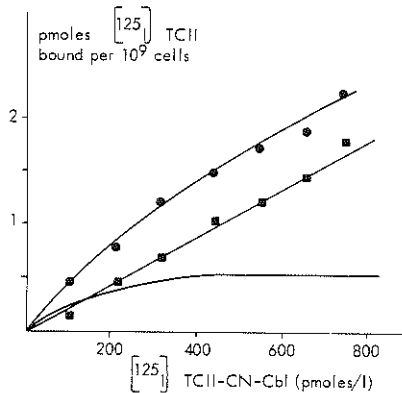function (3), also had no effect on the binding, uptake and

fig. 6.9. The initial binding of $\left[^{125}I\right]$ TC II-CN-Cbl as a
         function of the concentration of $\left[^{125}I\right]$ TC II-CN-
         Cbl. Measurements were made in the absence (●——●)
         and in the presence (■——■) of 10 nM unlabeled
         human TC II-CN-Cbl. A curve of saturable specific
         binding was obtained by subtraction of the second
         from the first (top) curve (————).

degradation of TC II-CN-Cbl. Mannose-6-phosphate, a known
competitive inhibitor of the uptake process in the secretion-
recapture mechanism of lysosomal hydrolases in fibroblasts
(4), did not influence the binding, uptake and degradation of
TC II. This makes it unlikely that the ligand is recognised
by the binding site through a phosphorylated residue on the
TC II molecule.

     On the contrary, chloroquine inhibited the degradation
of $\left[^{125}I\right]$ TC II and caused a compensatory accumulation of
$\left[^{125}I\right]$ TC II in the cell (figure 6.10). The binding remained
unchanged but the degradation was inhibited to about 50% at
the concentration of chloroquine used.
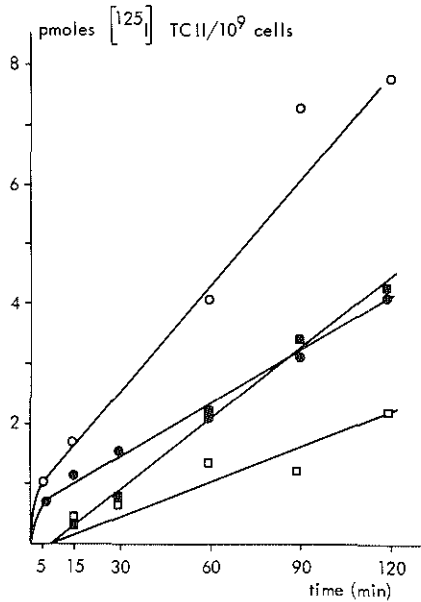
fig. 6.10. *Effect of 3.5 mM chloroquine on the binding,*
uptake and degradation of $\left[^{125}I\right]$ *TC II-CN-Cbl.*
The concentration of $\left[^{125}I\right]$ *TC II-CN-Cbl was*
400 pM. Each point is the mean of duplicate
measurements. ●————●,*binding and uptake in the*
control incubation; ■——■,*production of acid*
soluble material by the control cells. ○————○,
binding and uptake in the presence of chloroquine;
□————□,*production of acid soluble material in the*
presence of chloroquine.

6.5. Discussion.

The time course of the binding, uptake and degradation
shows that the TC II-Cbl complex enters the cell in a two-
phase process, a rapid binding onto the outer cell membrane
followed by a slow but steady uptake of the complex into the
cell. The binding step is not much influenced by the
temperature in contrast to the uptake. The appearance of acid

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soluble ^{125}I-labeled material in the medium indicates that
the entered TC II is degraded by proteolytic enzymes in the
lysosomes. At 4°C such degradation products were completely
absent, which indicates that it is not an elution of the
[125]I-label. The possibility of extracellular degradation as
a result of the release of the lysosomal enzymes from dead
or leaky cells in the medium was excluded by the absence of
proteolysis during incubation of [^{125}I]TC II-Cbl in the pre-
sence of sonicated cells at pH 7.4. Proteolysis of TC II
only occurred, when the pH of the disrupted cell suspension
was lowered to 5-6. It is highly unlikely that extracellular
proteolysis occurred in our incubations because the pH was
kept well at pH 7.4 during the incubation. The degradation
of [^{125}I]TC II during the incubation is therefore a process
which follows the uptake of the complex into the cell. This
assumption is supported by the observed effects of NH_4Cl and
chloroquine on the uptake and degradation of [^{125}I]TC II-Cbl.
Both chloroquine and NH_4Cl inhibit proteolysis in the lyso-
somal system presumably by an intralysosomal increase of the
pH (1,5). It has been reported that 0.05 mM chloroquine
completely inhibits proteolytic degradation of [^{125}I]TC II (6),
low-density lipoprotein (7) and cellular protein (8) in cul-
tured fibroblasts. In our liver cell system 0.05 mM chloro-
quine had no significant effect on uptake and degradation of
TC II, but at 3.5 mM chloroquine degradation of TC II
decreased considerably and consequently [^{125}I]TC II accumu-
lated in the cells. The necessity of high concentrations of
chloroquine in an isolated liver cell system has also been
reported by Nakai et al. (9) in their studies on the uptake
and degradation of ^{125}I-labeled high-density lipoprotein and
by Tolleshaug et al. (10), who obtained about 75% reduction
of the degradation of ^{125}I-labeled asialo-fetuin with 1 mM
chloroquine.

Regarding the specificity and saturability of the
uptake first the exchangeability of human and rat TC II-CN-

Cbl was investigated. Our experimental results indicated that human and rat TC II-CN-Cbl compete for the same binding sites and for the same mechanism of uptake, but also that a part of the binding and of the uptake is non-competitive. It proved possible to differentiate between competitive and non-competitive binding, uptake and degradation by incubations at various concentrations of $[^{125}I]$TC II-Cbl in the presence and absence of an excess of unlabeled human TC II-CN-Cbl. From the results it was concluded that the rate of competitive uptake was directly dependent on the degree of occupation of the specific binding sites. The difference between the TC II concentrations, at which half-maximal specific binding and uptake plus degradation was reached, 130 pM and 220 pM, is too small to be used as an argument against this conclusion. The non-competitive uptake is probably the result of non-specific, non-saturable binding to the cell membrane, which otherwise does not mean that specifically and non-specifically bound TC II molecules are internalized in different ways.

Some TC II will be taken up without previous membrane binding by bulk fluid endocytosis. The maximum rate of bulk fluid endocytosis observed in cultured fibroblasts was 0.0035% per hour of the administered concentration of marker per 10^6 cells (11). Even if the rate of pinocytosis in rat liver cells is much higher than in fibroblasts, it would not be sufficient to explain the observed rates of non-specific uptake. It remains to be established whether or not the non-specific uptake plays an important role in vivo, also in view of the very low concentration of TC II in relation to other plasma proteins.

The studies on the effects of EDTA and EGTA on the binding and uptake confirmed the observations reported in chapters 3 and 4, that calcium is not needed in the different phases of the uptake mechanism. The quantitative data which were obtained with iodinated TC II-CN-Cbl correspond well with the data from similar experiments with TC II-$[^{57}Co]$Cbl,

especially after correction for the non-specific processes. The non-specific binding and uptake with $[^{125}I]$TC II-Cbl were higher than in corresponding experiments with partially purified TC II-$[^{57}Co]$CN-Cbl, which may be due to the protein labeling procedure.

In conclusion the data from the binding, uptake and degradation studies are in agreement with the earlier proposed model of adsorptive endocytosis, followed by lysosomal degradation of the transport protein.

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GENERAL DISCUSSION

The purpose of the investigations described in this
thesis was to obtain a comprehensive picture of the vitamin
B_{12} uptake by the liver cell. This process can be divided
into three aspects, the interaction between the transcobala-
min II-vitamin B_{12} complex and the plasma membrane of the
cell, the transport of the vitamin to the inside of the cell
and the handling of the vitamin and its transport protein
within the cell.

As stated in chapter 3 the high association constant of
the TC II-Cbl complex and the rapid clearance of free vita-
min B_{12} by the kidneys make it very unlikely that significant
amounts of free vitamin B_{12} are present in the plasma and
will be taken up by the cells. The plasma transport protein,
transcobalamin II, is indispensable for an efficient uptake.
The severe megaloblastic anemia which has been observed in
patients with a congenital TC II-deficiency, has confirmed
this concept.

Our first efforts were directed to the demonstration of
specific binding sites on the plasma membrane of the rat
liver. Sucrose- density gradient centrifugation of a liver
homogenate fraction, which was rich in plasma membranes,
showed that the profile of the TC II-$[^{57}Co]$CN-Cbl distribu-
tion over the gradient paralleled the distribution of the
marker enzyme for plasma membranes. From this it was conclu-
ded that these plasma membranes indeed contained binding
sites for the complex. The affinity constant and the speci-
fic number of binding sites of isolated plasma membranes
were determined and the results agreed well with similar
studies in the literature (1).

The maximal specific binding of TC II-$[^{57}Co]$CN-Cbl to
the plasma membranes was 0.025 pmole per mg of membrane

protein and can be expressed as 0.8 pmole per 10^9 cells, as-
suming that 2% of the total liver protein is located in the
plasma membranes and that 1 g liver contains about 1.25×10^8
cells (2,3). The maximal specific binding of TC II-$\left[^{57}Co\right]$CN-
Cbl and $\left[^{125}I\right]$TC II-CN-Cbl to the isolated liver parenchymal
cells were 0.7 and 0.5 pmole/10^9 cells respectively. These
values agree well with each other and also with the value
obtained with isolated plasma membranes. Moreover the affini-
ty constants of the binding to the plasma membranes ($5-12 \times$
10^9 M^{-1}) and to the liver parenchymal cells ($6-7 \times 10^9$ M^{-1})
are in good agreement with each other. A similar affinity
constant (5.5×10^9 M^{-1}) has been described for the binding
of TC II-$\left[^{57}Co\right]$CN-Cbl to L 1210 lymphoblasts (4). Maximal
binding to the lymphoblasts was 0.7 pmole per 10^9 cells. In
cultured fibroblasts a higher affinity constant, 2×10^{10} M^{-1},
has been found and the maximal binding was about ten times
higher (5).

Unsaturated transcobalamin II (apo-TC II) did not com-
pete with the binding of saturated TC II (holo-TC II) to the
liver plasma membranes. Several authors have reported that
(pre)incubation of cells or membrane fractions with apo-TC II
inhibited the binding or uptake of the labeled holo-TC II (1,
6). Recently Hall and Green described that the uptake of
TC II-vitamin B_{12} by Hela-cells and lymphocytes was unaffec-
ted by apo-TC II , but that the binding of holo-TC II, measu-
red at $4^{\circ}C$, was competitively inhibited by apo-TC II (7). They
suggested that, although apo-TC II and holo-TC II compete
for the same binding sites on the plasma membrane, different
mechanisms exist for their entrance into the cell. Because
of the differences in the experimental conditions in the
various studies it is not possible to decide for or against
competitive inhibition. Our results, which are partially
supported by the observations of Hall and Green (7), suit
the physiological circumstances better, because the excess
of apo-TC II should not be able to block the uptake of holo-
TC II by the cell.

The second aspect of the vitamin B_{12} uptake, the trans-
port of the vitamin molecule into the cell, was studied with
isolated liver cells. The uptake and the binding consisted of
a specific and a non-specific component. Because it is not
clear to what extent the non-specific processes occur in vivo,
we will confine ourselves to the data on the specific satu-
rable uptake.

The rate of specific uptake of TC II-[57Co]CN-Cbl at
saturation was 1.1 pmole/10^9 cells/hour and of [125I]TC II-
CN-Cbl amounted to about 0.7 pmole/10^9 cells/hour. However,
these values do not include the radioactivity which had been
taken up and released again. For TC II-[57Co]CN-Cbl an esti-
mate of the total uptake can be obtained from the incubations
in the presence of chloroquine. The rate of uptake at 400 pM
TC II-[57Co]CN-Cbl was roughly 2.6 times the uptake in the
absence of chloroquine. Assuming that the effects of chloro-
quine in specific and non-specific uptake are the same, the
real rate of specific uptake of TC II-[57Co]CN-Cbl would be
about 2.9 pmoles/10^9 cells per hour. This value should
correspond with the rate of specific uptake plus degradation
of [125I]TC II-CN-Cbl at saturation, which was 1.2 pmole/10^9
cells per hour. The latter value is somewhat lower but diffe-
rences in the individual cell preparations may account for
this. Consequently the uptake plus degradation of [125I]TC II-
CN-Cbl in the presence and in the absence of chloroquine has
to be rougly the same. In fact they were 4.4 and 3.9 pmoles/
10^9 cells respectively after one hour of incubation in the
same cell population, which agrees with the presupposition.

The maximal rates of specific uptake in the liver cells
are lower than the the comparable values for L 1210 lympho-
blasts. DiGirolamo and Huennekens (4) observed a maximal
uptake of 0.4 pmole TC II-[57Co]CN-Cbl/10^9 cells per min and
Ostroy and Gams (8) reported a value of 18 pmoles/10^9 cells
per min. In fibroblasts the kinetics of the uptake have not
been analysed, but it is possible to deduce from the figures
that after an incubation of 6 hours about 5 pmoles/10^9 cells

are associated with the cell, of which 25% is bound to the surface of the cells, and 5-6 pmoles are secreted as small molecular weight degradation products (5).

On the basis of the data on binding, uptake and degradation of TC II-CN-Cbl it was attempted to construct a model for the mechanism of uptake. In chapter 3 three different models have been proposed:

1. the vitamin molecule enters the cytoplasm of the cell through the membrane after dissociation from its transport protein on the surface of the cell.
2. the whole TC II-vitamin B_{12} complex enters the cytoplasm of the cell through the membrane.
3. the TC II-vitamin B_{12} complex is taken up by the cell in a pinocytotic process.

There are no arguments in favour of the first model. Several authors have demonstrated that the TC II molecule is consumed during the uptake process and that re-utilization, analogous to e.g. transferrin, does not occur (6,9). Re-utilization would be a logical consequence of this model.

The second model is supported by Gams and coworkers, who presented three arguments in favour of this model: 1. the presence of TC II-vitamin B_{12} in the cytoplasm of L 1210 lymphoblasts (10); 2. a TC II dependent uptake of vitamin B_{12} by isolated mitochondria (11); 3. the kinetics of the uptake of TC II-Cbl by L 1210 lymphoblasts, which showed in their studies no separate processes of binding and uptake but a smooth continuous increase with time of cell associated radioactivity (8). An argument against a TC II-mediated uptake by mitochondria is the passive but specific uptake mechanism for free OH-Cbl in isolated mitochondria, which has been reported by Fenton et al. (12). They concluded that the concentration of vitamin B_{12} in the mitochondria takes place as a result of the binding to an intramitochondrial protein.

With the same L 1210 lymphoblasts DiGirolamo and Huennekens (4) did find a biphasic pattern of binding and uptake. The affinity constant of the binding of TC II-Cbl to the lymphoblasts was similar to the affinity constant of the

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binding to the isolated liver cells in our study. However, half-maximal binding and half-maximal uptake did not occur at the same concentration of TC II-Cbl. This observation may indicate that in the lymphoblasts the binding is not functionally linked to the uptake, thereby precluding a process of pinocytosis, or that the extracellular concentration of TC II-Cbl has an effect on the efficiency of internalization of the TC II-Cbl complex through pinocytosis or on the rate of pinocytosis itself.

The third model, involving binding to the plasma membrane, pinocytosis and lysosomal degradation, has been supported by in vivo experiments of Schneider et al. (6), who demonstrated that in the rabbit $[^{125}I]$TC II-Cbl is broken down by the tissues. Further evidence for this model has been presented by the experiments of Youngdahl-Turner (5), which demonstrated that internalized TC II was degraded in the lysosomes of cultured fibroblasts.

Our experimental results are in best accordance with the third model. First of all we have demonstrated that TC II is broken down by proteolysis in the lysosomes and the usual way for an extracellular protein to arrive in the lysosomes is by way of an endocytic process (13). Second, chloroquine inhibited the degradation of TC II and caused a compensatory accumulation of TC II and of Cbl in the cell, probably in the lysosomes. This observation precludes the possibility that TC II is broken down in the lysosomes after it has delivered its vitamin molecule to, for instance, the mitochondria. In the third place the kinetics of binding and uptake suggest a mechanism of pinocytosis. Half-maximal binding and half-maximal uptake, and saturation of binding and of uptake occur at about the same extracellular concentration of TC II-Cbl, which indicates that the degree of occupation of the membrane binding sites primarily determines the rate of uptake of the TC II-Cbl complex.

Analysing the kinetics of TC II-Cbl uptake by the liver cells it was assumed that the rate of formation of pinocyto-

tic vesicles is constant during the incubation and not affec-
ted by the binding of the ligand to the binding site. In the
literature there is evidence that the ingestion of particles
by a pinocytotic process is enhanced by the binding of the
particle to the membrane and that bivalent or multivalent
macromolecules, such as concanavalin A and immunoglobulins,
stimulate the rate of pinocytosis by their binding to the
cell surface. When the same macromolecules were made uni-
valent, stimulation was absent although the binding was not
affected. It is therefore unlikely that the TC II molecule,
which is thought to be univalent, is able to influence the
rate of pinocytosis of the liver cells.

A consequence of the quantitative data of binding and
uptake by the liver cell is, that each membrane binding site
allows the entrance of 2-4 TC II-Cbl molecules per hour.
Assuming that the binding sites are evenly distributed over
the total cell surface, and that no direct relation exists
between the site of pinocytosis and the localization of the
binding sites, a membrane turnover of 2-4 times per hour
would be necessary, as a result of the pinocytotic process,
to reach the observed rate of uptake. The rate of pinocyto-
sis in the liver cells, which was measured by plasma membrane
protein turnover studies by Schimke (14), appears to be only
25% of the plasma membrane surface per hour. Two different
explanations for this discrepancy between membrane turnover
and rate of uptake of extracellular protein have been given
by Neville and Chang (15): first the occurrence of receptor
clustering at the site of pinocytosis and second a high de-
gree of re-utilization of membrane components, which means
that the rates of plasma membrane protein turnover cannot be
used as a measure for rates of pinocytosis. So far no con-
vincing evidence has been given for any of the two models,
although Anderson et al. (16) concluded from electron
microscopic studies on the binding of ferritin-conjugated
low-density lipoprotein (LDL) to fibroblasts, that the LDL-
binding sites were concentrated in so-called coated regions,

figure 7.1.

CLUSTERED BINDING-SITE-MEDIATED PINOCYTOTIC TRANSPORT MODEL FOR
TRANSCOBALAMIN II - VITAMIN B$_{12}$ IN THE LIVER PARENCHYMAL CELL

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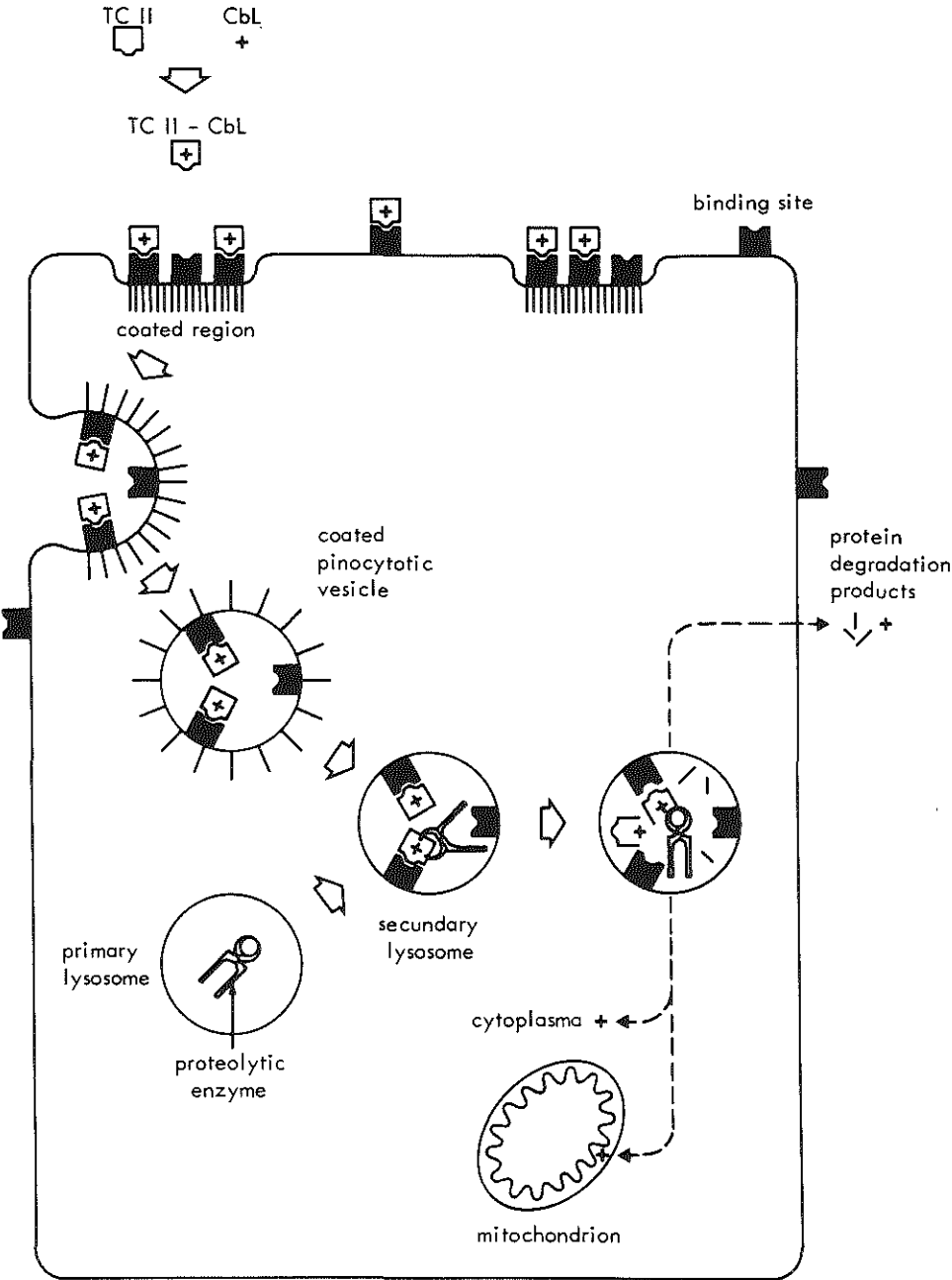
which accounted for only 1.4% of the total membrane surface area.

Clustering of the binding sites for TC II on the liver plasma membrane could explain the observed rates of uptake. Consequently the scheme for the uptake of TC II-vitamin B_{12} might be as illustrated in figure 7.1. First the TC II-vitamin B_{12} complex adheres to the clustered binding sites on coated regions in the plasma membrane, which are predestined to become sites of pinocytosis. After formation of the pinocytotic vesicle fusion with the primary lysosomes occurs and a secondary lysosome is formed. Meanwhile the TC II molecule is degraded by the lysosomal proteolytic enzymes and the vitamin B_{12} molecule is detached from its binding protein. The vitamin B_{12} molecule is probably transfered to the cytoplasm and the mitochondria and will be converted to the respective coenzyme forms. A large part of the internalized vitamin is released by the cell, possibly after conversion into biologically active forms.

In conclusion the mechanism of uptake of transcobalamin-II-vitamin B_{12} by the liver cell can be described as adsorptive endocytosis followed by lysosomal fusion and proteolytic degradation of the transport protein. Quantitative aspects of the kinetics of binding and uptake suggest a clustering of the binding sites in small areas of the plasma membrane. From the kinetic analysis of binding, uptake and release we concluded that at a normal TC II-Cbl concentration in the blood plasma the majority of the binding sites on the liver plasma membranes are occupied with holo-TC II and that TC II-Cbl is continuously taken up and released. A small fraction of the internalized vitamin B_{12} is probably retained by the cell to compensate for the continuous release of vitamin from the storage pool. In our studies no arguments have been found that the exchange between intra- and extracellular vitamin B_{12} is regulated by the cell on the basis of the extracellular concentration of TC II-vitamin B_{12}.

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SUMMARY

The object of the experiments, described in this thesis,
was to obtain a comprehensive picture of the uptake of
vitamin B_{12} by the liver cell, and of the handling of the
vitamin and its plasma transport protein, transcobalamin II,
in the cell. Chapter 1 gives a synopsis of the literature
on vitamin B_{12} transport and metabolism and describes the
different models which have been proposed for the mechanism
of the vitamin B_{12} uptake by the cell. In chapter 2 the
various techniques are described, which have been used
throughout the investigations. The first aspect of the
uptake process, the binding of the TC II-vitamin B_{12} complex
to the cell membrane, is treated in chapter 3. The presence
of specific binding sites on the isolated liver plasma
membrane was demonstrated and the kinetic aspects of the
binding process were studied. An affinity constant of
$5-10 \times 10^9$ M^{-1} was determined, which suggests that in vivo
the majority of the binding sites on the cell surface is
occupied by TC II-vitamin B_{12} at a normal plasma TC II-
vitamin B_{12} concentration of about 500 pM. Free vitamin B_{12}
and apo-TC II had no competitive effect on the binding of
holo-TC II. In contrast with the binding of TC II-vitamin
B_{12} to various other cells, the binding to the liver cell
membranes did not depend on the presence of calcium or
magnesium ions. It appeared to be possible to reduce the
binding capacity of the plasma membranes by preincubation
either with trypsin or with detergents, which indicates that
an essential part of the binding sites is composed of protein.
The phase which follows the binding of the complex to
the binding sites is its transfer to the interior of the
cell. This is discussed in the chapters 4 and 6. In chapter
4 experiments are described which were carried out with a
TC II-vitamin B_{12} complex, [57]Co-labeled in the vitamin
moiety, and in chapter 6 the fate of the transport protein

is studied with radioactive iodine labeled TC II-vitamin B_{12}. The incorporation of the radioactive label in the protein moiety of the complex necessitated the purification of TC II to homogeneity. For this purpose an affinity chromatography technique was developed, which is easy to perform and apparently does not harm the protein structure. 2.7 mg of TC II-CN-Cbl were purified from 20 kg of Cohn fraction III, prepared from 500 l human plasma. The final yield was about 50% and the specific binding capacity amounted to 0.98 mol CN-Cbl per mol TC II.

Studies on the cellular uptake were performed by incubations of TC II-CN-Cbl complex with isolated liver parenchymal cells in suspension. During the incubations a fast increase of cell-bound radioactivity was observed in the first few minutes and this phase was followed by a steady linear increase in the next two hours. This pattern suggests a rapid binding of the complex onto the surface of the cell and a relatively slow internalization of the TC II-Cbl complex. Both binding and uptake were partially saturable with increasing extracellular TC II-CN-Cbl concentrations. The assumption was made that the observed uptake is the sum of a specific and a non-specific process, which was confirmed by the results of incubations in the presence of an excess of unlabeled TC II-CN-Cbl. Under these circumstances the specific binding and uptake form only a small, negigible fraction of the total uptake,and the main uptake is the result of the non-specific process. The specific saturable binding and uptake were calculated by subtraction of the non-specific from the total binding and uptake.

Binding and uptake reached half-maximal levels and saturation at about the same concentrations of TC II-CN-Cbl, which suggests that the rate of uptake is primarily determined by the degree of occupation of the available binding sites on the cell membrane.

The uptake of TC II is accompanied by the formation and secretion of small molecular weight degradation products of

the TC II molecule. Addition of chloroquine, a known inhibi-
tor of intralysosomal proteolytic enzymes, to the incubation
system led to an accumulation of the transport protein and
the vitamin within the cell and to a reduction of proteolysis
of TC II-Cbl. This suggests that proteolysis of TC II in the
lysosomes is a prerequisite for the normal cellular handling
of vitamin B_{12}. Moreover, it indicates that the observed
uptake of vitamin B_{12} is not representative for the total
uptake but only the resultant of a process of uptake and of
release. This conclusion was confirmed by observations on the
release of vitamin B_{12} by isolated liver cells which were
loaded with radioactive vitamin B_{12} shortly before their iso-
lation. These experiments suggest that vitamin B_{12} which is
bound to the surface of the cells or has been taken up in the
pinocytotic vesicles and in the lysosomes, can be released
completely by the liver cells during short incubations.
However, when the radioactive vitamin B_{12} was administered
70 hours before the isolation of the liver cells, only 15%
of the radioactive vitamin was released during two hours of
incubation. The same percentage was found when the release
of total intracellular vitamin B_{12} into the medium was deter-
mined. This observation indicates that after 70 bours the
internalized vitamin B_{12} is taken up in the vitamin B_{12}
storage pool of the liver.

Summarizing all qualitative and quantitative data from
the various experiments the whole rat liver is able to bind
0.5 to 0.7 pmole and to internalize about 2.3 pmoles of TC II-
CN-Cbl per hour from which about 2/3 is released again.
Concomittantly the transport protein is degraded in the lyso-
somes and secreted as small molecular weight degradation
products. This suggests that TC II-vitamin B_{12} is internali-
zed by means of adsorptive endocytosis or binding site- media-
ted pinocytosis, followed by fusion of the endocytic vesicles
withe the lysosomal system.

On the basis of the relation between the amount of bin-
ding sites and the observed rate of uptake each binding site

should be able to internalize 2-4 molecules of TC II-Cbl per
hour. In terms of plasma membrane turnover rates this impli-
cates that either the binding sites are subject to a high
degree of re-utilization or the binding sites are mainly con-
centrated in small areas of the cell surface or both.
Concentration of binding sites in so-called coated regions
have been described earlier for low-density lipoprotein recep-
tors on fibroblast membranes. The mechanism of the uptake of
TC II-Cbl by the liver parenchymal cell can therefore most
likely be defined as clustered binding site- mediated pino-
cytosis.

SAMENVATTING

De experimenten waarvan de resultaten in dit proef-
schrift zijn beschreven hadden tot doel een samenvattend
beeld te krijgen van de opname van vitamine B_{12} vanuit het
plasma door de levercel en van de verdere verwerking binnen
die cel. In hoofdstuk 1 wordt een overzicht gegeven van de
historische ontwikkelingen in het onderzoek naar het
vitamine B_{12} transport en metabolisme in het algemeen en
van de cellulaire opname processen in het bijzonder.
Ook de isolatie en karakterisatie van het plasma transport-
eiwit, transcobalamine II, krijgen ruim aandacht. Uit die
vele gegevens, afkomstig van experimenten met verschillende
celtypen en onder diverse experimentele omstandigheden,werden
de volgende conclusies getrokken:
1. Transcobalamine II is een vitamine B_{12} bindend plasma-
   eiwit met een moleculairgewicht van 38 000, dat een onmis-
   bare rol vervult in de opname van vitamine B_{12} door de
   cel.
2. Op de buitenmembraan van de cel bevinden zich bindings-
   plaatsen voor het transcobalamine II-vitamine B_{12} complex.
3. Het transcobalamine II-vitamine B_{12} complex wordt in zijn
   geheel door de cel opgenomen en het transporteiwit gaat
   bij dit proces verloren, vermoedelijk door proteolytische
   afbraak in de lysosomen.
4. De opgenomen vitamine wordt voor een deel weer vrij snel
   door de cel uitgescheiden. In fibroblasten is dit ten dele
   afhankelijk van de aanwezigheid van een intracellulair
   vitamine B_{12} bindend eiwit.

De literatuurgegevens zijn op een aantal punten min of meer
met elkaar in tegenspraak. Dat betreft ten eerste de
calciumafhankelijkheid van de binding van het transcobalamine
II-vitamine B_{12} complex aan de bindingsplaatsen op de plasma-
membraan. Ten tweede worden tegenstrijdige resultaten

beschreven aangaande het competitieve effect van onverzadigd
of apo-transcobalamine II ten opzichte van verzadigd of
holo-TC II. Een derde controverse kan worden waargenomen
in de beschrijving van de intracellulaire verwerking van
het TC II-vitamine B_{12} complex, waarin het model van lyso-
somale afbraak van TC II, gevolgd door intrede van de
vrije vitamine B_{12} molecule in het cytoplasma, en het model
van direct transport van het intacte complex naar het cyto-
plasma, gevolgd door een TC II afhankelijke opname van de
vitamine B_{12} molecule door de mitochondriën, tegenover
elkaar staan.

In het nu volgende overzicht van de resultaten uit dit
proefschrift wordt duidelijk gemaakt aan welke karakteris-
tieken de opname door de levercel voldoet.

In hoofdstuk 3 wordt aannemelijk gemaakt dat lever-
cellen op hun plasmamembraan specifieke bindingsplaatsen
bezitten. De affiniteit van transcobalamine II-vitamine B_{12}
ten opzichte van deze bindingsplaatsen ligt in de orde van
5 à 10 x 10^9 M^{-1}. Deze waarde doet vermoeden dat bij een
plasma concentratie aan TC II-vitamine B_{12} van $\pm$ 500 pM
het merendeel van deze bindingsplaatsen op de membraan bezet
is. De affiniteit van de bindingsplaatsen in diverse
weefsels vertoont weinig onderlinge verschillen. De binding
van TC II-vitamine B_{12} wordt niet beïnvloed door de aanwezig-
heid van vrije vitamine B_{12} of van apo-TC II en de binding
is niet afhankelijk van calcium- of magnesiumionen. Het bleek
mogelijk het aantal bindingsplaatsen sterk te reduceren door
incubaties van de membraanpreparaten met trypsine en met
detergentia. Hieruit mag de conclusie worden getrokken dat
een essentieel onderdeel van de bindingsplaats is opgebouwd
uit eiwit.

De fase, die volgt op de binding van het complex aan
de plasmamembraan, is het transport de cel in en wordt
beschreven in de hoofdstukken 4 en 6. Hoofdstuk 4 betreft
opnamestudies met geïsoleerde rattelever parenchymcellen,

die zijn uitgevoerd met een TC II-vitamine B_{12} complex dat radioactief gemerkt is in het vitamine B_{12} gedeelte. In hoofdstuk 6 worden vergelijkbare experimenten vermeld die zijn uitgevoerd met de radioactieve merking in het eiwit-gedeelte.

Tijdens incubatie van een levercel suspensie met het radioactief gemerkte TC II-vitamine B_{12} wordt in de eerste minuten een sterke stijging van celgebonden radioactiviteit waargenomen, en deze eerste fase wordt gevolgd door een tweede fase van geleidelijke lineaire toename van de cel-gebonden activiteit. Dit patroon suggereert een snelle binding van het TC II-vitamine B_{12} complex aan het cel-oppervlak en een relatief langzaam transport de cel in. Zowel binding als opname vertonen met stijgende TC II-vitamine B_{12} concentraties een gedeeltelijke verzadiging. Het vermoeden dat we hier te maken hebben met een combinatie van een specifiek en een niet-specifiek proces, wordt bevestigd door de resultaten van incubaties in aanwezigheid van een overmaat aan ongemerkt TC II-vitamine B_{12}. Onder die omstandigheden worden alleen de niet-specifieke binding en opname gemeten en deze blijken met toenemende gemerkte TC II-vitamine B_{12} concentraties lineair toe te nemen. Uit de combinatie van beide proefopzetten is het mogelijk de specifieke verzadigbare component van de binding en de opname afzonderlijk te berekenen. De overeenkomst tussen de concentratie-afhankelijkheid van de binding en van de opname geeft aan, dat de opnamesnelheid primair wordt bepaald door de bezettingsgraad van de beschikbare bindingsplaatsen op de celmembraan.

De opname van TC II gaat gepaard met de vorming en uitscheiding van klein-moleculaire afbraakprodukten van de TC II molecule. Toevoeging van chloroquine, een remmer van intralysosomale proteolytische enzymen, aan het incubatie systeem leidt tot een accumulatie van het transporteiwit en van de vitamine B_{12}. De som van opname en afbraak van

TC II wordt nauwelijks door chloroquine beïnvloed. Hieruit wordt de conclusie getrokken, dat de waargenomen opname van vitamine B_{12} in feite slechts de hoeveelheid vitamine B_{12} weergeeft die na opname in de cel achterblijft, en dat in afwezigheid van chloroquine het grootste deel van de opgenomen vitamine B_{12} de cel weer verlaat. Deze conclusie wordt bevestigd door experimenten, waarin de uitscheiding van vitamine B_{12} door geïsoleerde levercellen wordt bestudeerd, nadat deze cellen vóór hun isolatie in vivo zijn opgeladen met radioactief gemerkte vitamine B_{12}. Wanneer de in vivo toediening heeft plaatsgevonden 30 min voor de aanvang van de celisolatie, wordt radioactief gemerkte vitamine vrijwel volledig uitgescheiden gedurende een incubatie van 2 uur. Heeft de in vivo oplading 70 uur tevoren plaatsgevonden dan wordt slechts ± 15% gedurende een 2-uurs incubatie uitgescheiden.

Hoofdstuk 5 beschrijft de isolatie van transcobalamine II uit humaan plasma. Deze isolatie is een essentieel onderdeel van het totale onderzoek, omdat selectieve radioactieve merking van het transporteiwit slechts mogelijk is, indien het te merken materiaal volledig zuiver is. Om dit doel te bereiken werd een affiniteitschromatografie techniek ontwikkeld, die eenvoudig uitvoerbaar is, tot een efficiënte zuivering leidt en geen schadelijke invloed op het te zuiveren eiwit heeft. Uit 20 kg Cohn's fractie III van ± 500 l humaan plasma werd, met een opbrengst van 50%, 2,7 mg zuiver transcobalamine II met een specifieke binding van 0,98 mol CN-Cbl/mol TC II geïsoleerd. Door middel van electroforetische analyses van het gezuiverde produkt blijkt dat het enigszins heterogeen van samenstelling is. Het is mogelijk een deel van het materiaal door reductie met β-mercaptoethanol en dithiothreïtol in 2 sets van 2 complementaire fragmenten op te splitsen. Of de fragmenten natuurlijk voorkomende subunits zijn of dat zij het gevolg zijn van proteolyse of een andere invloed van buitenaf, valt uit

onze resultaten niet op te maken. Met behulp van iso-
electrische focussering van het zuivere eiwit blijkt het
mogelijk het eiwit te scheiden in op zijn minst vier
verschillende subfracties. Dit sluit aan bij recente
gegevens aangaande de genetische variabiliteit van trans-
cobalamine II.

In hoofdstuk 7 worden de resultaten van alle in dit
proefschrift beschreven experimenten aan elkaar getoetst en
als bouwstenen gebruikt voor een model van de opname van
vitamine B_{12} door de levercel. In dit model is de volgorde
van gebeurtenissen als volgt:
1. TC II-vitamine B_{12} bindt zich aan specifieke bindings-
   plaatsen op het celoppervlak, die op grond van
   kwantitatieve gegevens waarschijnlijk in zogenaamde
   "clusters" geconcentreerd zijn.
2. Met een gedeelte van de membraan wordt het hele complex
   via pinocytose de cel binnengevoerd.
3. Het pinocytose-blaasje vervloeit met het lysosomale
   systeem, waarna door inwerking van lysosomale proteoly-
   tische enzymen het transporteiwit wordt afgebroken en
   vitamine B_{12} wordt vrijgemaakt.
4. De vitamine B_{12} molecule komt via onbekende wegen terecht
   in het cytoplasma en het mitochondrion of wordt door de
   cel uitgescheiden.

Transcobalamine II schaart zich daarmee in de rij van ei-
witten, waarvoor een vergelijkbaar opnamemechanisme reeds
eerder werd waargenomen, zoals asialoglycoproteinen, "high-
and low-density" lipoproteine en lysosomale hydrolases
in fibroblasten.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 18 september 1949 geboren te Rotterdam. Hij behaalde in 1967 het diploma Gymnasium β aan het Libanon Lyceum te Rotterdam en begon in hetzelfde jaar de studie in de Biologie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen B4 werd afgelegd in juni 1970 en het doctoraalexamen met de hoofdvakken biofysische chemie en hematologie en het bijvak biochemie (cum laude) in november 1972.

Sinds 1 december 1972 is hij als wetenschappelijk medewerker verbonden aan het Instituut voor Hematologie van de Erasmus Universiteit Rotterdam. Aldaar werd onder leiding van Prof.Dr. J. Abels het hier beschreven onderzoek verricht.

Op de omslag is een foto weergegeven van
geïsoleerde rattelever parenchymcellen, gezien
door een fase-contrastmicroscoop.