Subcellular localisation of vitamin $\text{B}_{12}$ during absorption in the guinea-pig ileum*

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SUMMARY The subcellular distribution of vitamin $\text{B}_{12}$ was studied during its absorption in the guinea-pig ileum. Animals were fed either ($^{57}\text{Co}$) or ($^{58}\text{Co}$) cyanocobalamin and killed two or four hours later. At two hours labelled cyanocobalamin was concentrated in brush border and lysosomal fractions of ileal homogenates, with some remaining in the sample layer. In contrast, at four hours labelled cyanocobalamin was concentrated predominantly in the cytosol with much smaller peaks in the brush border and lysosomal fractions. These findings are consistent with vitamin $\text{B}_{12}$ absorption by receptor-mediated endocytosis. It is suggested that, after binding at the brush border, vitamin $\text{B}_{12}$ is first sequestrated within lysosomes, and then released into the cytosol, from where it leaves the cell to enter the portal blood.

During the absorption of vitamin $\text{B}_{12}$ ($\text{B}_{12}$) in the ileum, intrinsic factor-vitamin $\text{B}_{12}$ (IF-$\text{B}_{12}$) complexes bind to specific receptors on the microvillous membranes of ileal enterocytes. There is then a significant delay of about two hours before $\text{B}_{12}$ appears in the portal blood. Neither the cause for this delay, nor the pathway that $\text{B}_{12}$ takes in its passage across the enterocyte is known. Two hypotheses have been advanced. As IF has not been found in portal blood, both involve cleavage of the IF-$\text{B}_{12}$ complex. Wilson first suggested that the IF-$\text{B}_{12}$ complex is absorbed by pinocytosis, and that cleavage occurs during this process. It has since been shown that in many mammalian cells pinocytic vesicles fuse with primary lysosomes to form secondary lysosomes. Lysosomes contain powerful hydrolytic enzymes, and these might cleave the IF-$\text{B}_{12}$ complex, and so release $\text{B}_{12}$ for transport into plasma. The other hypothesis is that of MacKenzie and Donaldson, who suggested that the IF-$\text{B}_{12}$ complex is split to an 'active fragment' of the $\text{B}_{12}$ molecule by brush border hydrolases, and that this smaller fragment is then transported into the cell, where $\text{B}_{12}$ is re-synthesised. However, since advancing this hypothesis, Donaldson has reported evidence that intrinsic factor may be taken up by isolated ileal enterocytes.

Previous studies in which subcellular fractionation techniques were used to investigate $\text{B}_{12}$ absorption in the ileum have not provided evidence which supports either view, but have apparently demonstrated concentration of absorbed $\text{B}_{12}$ in the mitochondria and microsomes of ileal enterocytes. This paper reports further subcellular fractionation studies performed by isopycnic centrifugation of guinea-pig ileal homogenates after the gastric administration of ($^{57}\text{Co}$) or ($^{58}\text{Co}$) cyanocobalamin.

Methods

ANIMALS Non-pregnant female guinea-pigs (Hartley strain) weighing between 500 and 600 g were used in all studies. After an overnight fast, they were lightly anaesthetised with halothane to permit peroral gastric intubation, and 0.25 ml cyanocobalamin solution radiolabelled with $^{57}\text{Co}$ or $^{58}\text{Co}$ was introduced into the stomach. This was followed by 1.5 ml water to wash out the tube contents. In the first set of experiments six guinea-pigs were given 10 ng ($^{57}\text{Co}$) cyanocobalamin (S.A. 8.14 MBq/µg) at the start of the study, and the animals were killed by a blow on the head two hours later. In the second set of experiments another six guinea-pigs were given 10 ng ($^{57}\text{Co}$) cyanocobalamin as before, and then 60 ng ($^{58}\text{Co}$) cyanocobalamin...
(S.A. 1.33 MBq/μg) at two hours. They were killed at four hours.

**HOMOGENISATION**

One hundred to one hundred and fifty milligram portions of ileum 5 cm proximal to the ileocaecal valve were excised immediately after death and cut into small pieces. They were homogenised by 12 strokes of a loose-fitting (type A) pestle in a glass Dounce homogeniser (Kontes Glass Co., Vineland, NJ, USA) in 4 ml 0.25 M sucrose containing 3 mM HEPES pH 7.4 (SH medium) at 4°C. The homogenate was centrifuged at 600 g for 10 minutes to remove nuclei, intact cells, and debris. The pellet was resuspended in another 2 ml SH medium by three strokes of the type A pestle, and centrifuged again.

**SUBCELLULAR FRACTIONATION**

Two millilitres of the combined postnuclear supernatants were layered on to a 20 ml preformed linear gradient of Percoll (Pharmacia (Great Britain) Ltd, Hounslow, England) in SH medium extending from a density of 1.025 to 1.15 g/ml. The gradients were centrifuged at 65 000 g for 30 minutes in an MSE 3 swing-out rotor (Measuring and Scientific Equipment, Crawley, Sussex, UK) at 4°C. Linearity of the gradients was preserved during this centrifugation and was reproducible. After tube piercing, 15 fractions were collected by upward displacement with 60% sucrose (w/v) into tared tubes. After weighing, the density of the fractions was determined indirectly by refractometry.

**ASSAYS**

The following enzymes were assayed in the gradient fractions and used as markers for subcellular organelles: alkaline phosphatase for the brush border; lactate dehydrogenase for the cytosol; β-N-acetyl-D-glucosaminidase for lysosomes; and glutamate dehydrogenase for mitochondria. Gradient fractions were counted for 57Co and 58Co in an LKB-Wallac 1280 Ultrogamma counter (LKB Produkter-AB, Bromma, Sweden) using mode 2 for double labelled samples with automatic background subtraction.

**PRESENTATION OF RESULTS**

All results are presented in the form of frequency-volume histograms. The calculations and plots were done by computer. Frequency is defined as the proportion of the total recovered activity found in the individual fraction divided by the fractional volume. All histograms are normalised, and the percentage recoveries (sum of gradient fractions against original postnuclear supernatant) are given in addition. Pooling and averaging of distributions were performed as described by Leighton et al.14 This method requires conversion of all the histograms to the same preset volume intervals, and causes some loss in resolution.

**Results**

Figure 1 shows the subcellular distribution of 57Co in guinea-pig ileal homogenates two hours after intragastric (57Co) cyanocobalamin. Although radioactivity is widely distributed within the gradient, it appears to be concentrated in three peaks, which correspond to the distribution of marker enzymes for the brush border and lysosomes with some activity remaining in the sample layer. This may represent radioactivity present in the cytosol in vivo, or may be due to release of (57Co) cyanocobalamin from organelles damaged during homogenisation. However, the latter possibility is unlikely to be very important, as lysosomal latency, which is an indirect and sensitive measure

![Subcellular fractionation of postnuclear supernatant from guinea-pig ileal homogenate two hours after intragastric (57Co) B12. Graph shows frequency-volume histograms for (57Co) B12, alkaline phosphatase, β-N-acetyl-D-glucosaminidase, glutamate dehydrogenase, and represent averaged results from six experiments. Frequency (mean±SD) is defined as the fraction of total recovered activity present in the individual fraction divided by the fractional volume. Percentage recoveries (mean±SD) (57Co) B12, 96±5; alkaline phosphatase, 103±10; β-N-acetyl-D-glucosaminidase, 76±8; glutamate dehydrogenase, 83±6; lactate dehydrogenase, 63±5.](http://gut.bmj.com/)

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of lysosomal integrity, and thus organelle damage, was high (70.7±3.3%, mean±SD) in our studies.

As radioactivity might have become associated with subcellular organelles during cell disruption and the subsequent centrifugation, an experiment was performed to investigate this possibility. Ileum from a control guinea-pig was homogenised in the presence of 10 ng ($^{57}$Co) cyanocobalamin, and then fractionated as above. Figure 2 shows that virtually all the radioactivity remains in the sample layer, and there is no evidence of concentration in any subcellular organelle. The same result was obtained when human intrinsic factor was added with ($^{57}$Co) cyanocobalamin.

The use of low concentrations of digitonin during homogenisation has been described to selectively disrupt lysosomes while leaving mitochondria intact. Figure 3 shows the effect of adding digitonin (0.12 mM final concentration) to the SH medium before homogenising ileum from a guinea-pig given intragastric ($^{57}$Co) cyanocobalamin two hours earlier. The lysosomes have been disrupted, so that β-N-acetyl-D-glucosaminidase activity remains mostly in the sample layer, where the radioactivity is also localised. In contrast, the distribution of glutamate dehydrogenase is unchanged from that shown in Fig. 1, indicating that the mitochondria remain intact. This makes it unlikely that vitamin B$_{12}$ is significantly concentrated in the mitochondria of the enterocytes during its absorption in the ileum.

In the second set of experiments ($^{57}$Co) cyanocobalamin was administered to the guinea-pigs as before, followed two hours later by ($^{58}$Co) cyanocobalamin. Figure 4 shows the subcellular distribution of $^{57}$Co and $^{58}$Co in ileal homogenates after another two hours. Although a small amount of $^{57}$Co given first is still found within the gradient, the majority is now recovered in the sample layer corresponding to a localisation in the cytosol. In contrast, the distribution of $^{58}$Co given later is similar to that shown in Fig. 1 for ($^{57}$Co) cyanocobalamin, which was also given two hours before killing in the first set of experiments. The experiments using both $^{57}$Co and $^{58}$Co labelled cyanocobalamin were repeated using the isotopes in reverse order. Whichever was given first was concentrated in the cytosol, and the isotope given later showed major peaks in the brush border and lysosomal fractions.

**Discussion**

Although we do not have direct evidence that radioactivity is associated only with cyanocobalamin, previous studies make it very unlikely that a
significant amount of $^{57}\text{Co}$ or $^{58}\text{Co}$ is detached from cobalamin under the conditions of these experiments.\textsuperscript{16} Our data show that two hours after feeding $^{57}\text{Co}$ or $^{58}\text{Co}$ cyanocobalamin to fasted guinea-pigs radioactivity is concentrated not only in the brush borders as might be expected, but also in lysosomal fractions of ileal homogenates (Figs. 1 and 4). However, the standard deviations shown for the distribution of radiolabelled cyanocobalamin are quite large. This is partly an inevitable consequence of the averaging process,\textsuperscript{14} which is necessary to permit presentation of results from several experiments, but causes some unavoidable loss of resolution. The other major limitation is that the time for the administered cyanocobalamin to pass from the stomach to the distal ileum may vary in individual animals, so that absorption may be at different stages in different animals even though they were all killed after the same interval. For this reason, it was decided not to study the change in subcellular distribution of absorbed $\text{B}_{12}$ with time by using several time points in different animals. Instead, we gave individual guinea-pigs first one radiolabelled cyanocobalamin followed after a set interval by a differently radiolabelled cyanocobalamin, and compared the difference in subcellular distribution of the two isotopes in ileal enterocytes. It was assumed that transit time from the stomach to the ileum was the same for both isotopes in individual guinea-pigs. The results of such experiments (Fig. 4) clearly show that at four hours there is much less of the absorbed ($^{57}\text{Co}$) cyanocobalamin in the brush border and lysosomal fractions, where it had previously been concentrated, but there is concentration of radioactivity in the cytosol.

We found no evidence of concentration of radioactivity in the mitochondria of ileal enterocytes during the absorption of ($^{57}\text{Co}$) cyanocobalamin. This contrasts with previous studies,\textsuperscript{9,10} and may be due to differences in the fractionation procedures employed. Peters and Hoffbrand\textsuperscript{9} demonstrated that two hours after oral ($^{57}\text{Co}$) cyanocobalamin radioactivity was concentrated in a combined mitochondrial and lysosomal fraction obtained by differential centrifugation of guinea-pig ileal homogenates. Additional fractionation of the combined mitochondrial and lysosomal fraction into partially purified mitochondria and lysosomes showed concentration in the former rather than the latter. However, no assessment of the purity of these fractions was made, and the maximum proportion of $^{57}\text{Co}$ in the combined mitochondrial and lysosomal fraction was 31%.

In contrast, approximately 50% of the total radioactivity was recovered in the soluble fraction at all times studied from 30 minutes to four hours. Rosenthal et al.,\textsuperscript{10} who also performed differential centrifugation of guinea-pig ileal homogenates, found that the cytosol contained very little $\text{B}_{12}$ during early absorption, but reached peak values four hours after dosage. This is similar to our findings. They also demonstrated transient concentration of $\text{B}_{12}$ in both mitochondrial and microsomal fractions maximal after 90 minutes and suggested that, during absorption, $\text{B}_{12}$ was first localised there, and then released into the cytosol in dialysable form. However, no attempt was made to separate the lysosomes, which would therefore have sedimented with the crude mitochondrial and microsomal fractions which they obtained.
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We found that the use of iso-osmotic gradients of Percoll in sucrose made possible rapid and reproducible subcellular fractionation of guinea-pig ileal enterocytes with minimal damage to the organelles, as demonstrated by the high lysosomal latency. Figure 3 shows that damaging the lysosomes, in this case by treatment with digitonin, causes release of their contents, which are recovered mainly in the soluble fraction. In previous studies of vitamin $B_{12}$ absorption it is possible that some mechanical disruption of the lysosomes may have occurred in the multiple centrifugation and resuspension procedures, and caused ($^{57}$Co) cyanocobalamin, which had been contained within the lysosomes in vivo, to be recovered in the soluble fraction.

We conclude that during the absorption of ($^{57}$Co) cyanocobalamin it is significantly concentrated in the lysosomes of the ileal enterocytes, and that concentration in the cytosol occurs later, so that the period of ileal delay corresponds to the intralysosomal phase. These conclusions are consistent with the process of receptor-mediated endocytosis, which has been described for the uptake of many receptor-bound proteins, including the uptake of transcobalamin II-bound $B_{12}$ by rat kidney and liver, and by human fibroblasts. We suggest that, after binding at the brush border, IF-$B_{12}$ is first sequestered in secondary lysosomes formed by the fusion of endocytic vesicles with primary lysosomes, and then $B_{12}$ is released into the cytosol, from where it leaves the cell to enter the portal blood bound to transcobalamin II.

What happens to IF is unknown. It may be degraded by lysosomal enzymes, which is the fate of many other transport proteins. This possibility may be elucidated by future studies with satisfactorily radiolabelled IF. IF appears to be internalised during vitamin $B_{12}$ absorption, as studies in which isolated guinea-pig ileal enterocytes were incubated with ($^{57}$Co) cyanocobalamin complexed with human IF show intracellular $^{57}$Co still associated with IF after 45 minutes. These results complement our own, and lend support to the suggestion that IF-$B_{12}$ is absorbed intact in the ileum by receptor-mediated endocytosis.

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