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Localization of vitamin B_{12} binding in *Euglena gracilis*

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Different fractionation procedures were used to determine the location of vitamin B_{12} binding sites in *Euglena gracilis*. Using uptake measurements, cell fractionation, and light and electron microscopy, the cuticle of the cell was found to be the fraction containing the majority of B_{12} binding sites. The apparent distribution of vitamin binding sites differed according to the cell lysis method used. The cuticle fraction was responsible for the binding of 80% of the vitamin taken up by the cell during both the rapid and the slow phase of uptake. These results suggest that vitamin B_{12} binding is regulated, in part, at the cuticle level, and support our previous conclusion that the secondary phase of uptake represents the synthesis of new receptor sites and not the unloading of vitamin inside the cell.

Introduction

Euglena gracilis cells are known to accumulate amounts of vitamin B_{12} well in excess of their requirements for growth (Varma *et al.*, 1961; Sarhan *et al.*, 1980). Vitamin B_{12} deficiency is associated with several metabolic and structural changes. During B_{12} deficiency, protein, RNA and DNA levels were found to increase (Bertaux & Valencia, 1971, 1972; Carell *et al.*, 1970). These changes were associated with modification of the nuclear (Bertaux *et al.*, 1978) and cuticular structure (Bré & Lefort-Tran, 1978; Lefort-Tran *et al.*, 1980). A block in cell division and an increase in cell volume were also observed (Epstein *et al.*, 1962; Bertaux & Valencia, 1973). A deficiency in vitamin B_{12} can also affect chloroplast replication (Lefort-Tran *et al.*, 1980; Carell, 1969; Christopher *et al.*, 1974). Previous reports have indicated that vitamin B_{12} bound to the chloroplastic (Sarhan *et al.*, 1980; Isegawa *et al.*, 1984), mitochondrial, microsomal, cytosolic (Isegawa *et al.*, 1984) and cuticle fractions (Varma *et al.*, 1961; Watanabe *et al.*, 1987). These discrepancies, which make it difficult to determine the location and the role of vitamin B_{12} in *E. gracilis*, could be the result of differences in cell fractionation procedures. In this study, using different cell lysis methods, light and electron microscopy and uptake measurements, we were able to determine that in *E. gracilis* the cuticle is the main site for vitamin B_{12} uptake during both the rapid and the slow phase of accumulation.

Methods

Cell culture, lysis, and fractionation. Euglena gracilis strain z (ATCC 12894) was used. Cell culture and uptake measurement of vitamin B_{12} were as previously described (Sarhan *et al.*, 1980). Cells were labelled with [^{57}Co]B_{12} (10 ng ml^{-1}; 0·004 μCi ng^{-1}, 148 Bq ng^{-1}; Amersham) in glutamate buffer (0·018 M-sodium glutamate pH 3·5, 2·5 mM-histidine.HCl, 2·2 mM-KH_2PO_4 and 0·056 M-glucose) for a period of 2 min or 4 h to follow the rapid and slow phase of uptake, respectively. Cells were immediately washed twice with ice-cold buffer to prevent possible displacement of the vitamin during the secondary uptake (Sarhan *et al.*, 1980). The wash buffer was either glutamate or Tris/HCl (0·15 M-Tris pH 7·5, 0·01 M-KCl, 1 mM-$MgCl_2$, 1 mM-EDTA and 0·25 M-glucose). Cells were then lysed, in either buffer system, at 4 °C under dim light to avoid the formation of reactive radicals, by two different procedures: sonication (Sarhan *et al.*, 1980), or breakage in a precooled mortar and pestle with glass beads 0·45 mm in diameter. The lysates were then centrifuged at 4000 *g* for 10 min. In all cases, the pellet was suspended in the respective lysate buffer and fractionated on a sucrose density gradient as previously described (Sarhan *et al.*, 1980). The 4000 *g* supernatant was centrifuged for 30 min at 26000 *g* to separate the bulk of mitochondria, which sedimented to the bottom of the tube. Polysomes were sedimented from the resulting supernatant by centrifugation at 195000 *g* for 90 min. The radioactivity distribution was estimated for each fraction by liquid scintillation counting as described previously (Sarhan *et al.*, 1980).

Light and electron microscopy. Preliminary identification of fractions separated on the sucrose gradients was performed with a Wild M-20 light microscope. Prior to electron microscopy, the fractions containing glass debris were centrifuged at 10000 *g* for 15 min over a 100% glycerol cushion. This treatment eliminated glass debris and nuclei while the radioactivity remained at the surface of the glycerol cushion. For electron microscopy observation, cell fractions were centrifuged and suspended in 2 ml 5% (w/v) glutaraldehyde and incubated for 2–4 h in the dark. After three washes with Tris buffer (pH 7·5), the fractions

were suspended for 1 h at 4 °C in 1·5% (w/v) osmium tetroxide. After centrifugation, the pellet was suspended in 1·5% (w/v) agar at 45 °C and immediately centrifuged in a benchtop centrifuge. The pellet in agar was sliced into 1 mm^3 blocks and dehydrated in an ethanol series (30, 50, 70, 80, 90 and 95%, v/v). The last three treatments were for 30 min in 100% ethanol. Fractions were then washed twice with 1 ml propylene oxide and partial embedding was done in 0·5 ml propylene/Epon (1:1, v/v) for 1 h. The mixture was then replaced with Epon and agitated for another 30 min. The blocks of agar were then transferred into moulds, new Epon added and polymerization allowed to proceed for 48 h at 60 °C. Samples were then prepared for electron microscopy by ultramicrotomy (LKB) and staining with 0·2% lead citrate for 2 min. Samples were then rinsed with 0·1 M-NaOH followed by distilled water prior to observation with a Philips EM-300 electron microscope.

Results and Discussion

The 4000 *g* pellet, which consisted of nuclei, cuticles, chloroplasts and paramylon granules, contained 80% of the labelled vitamin B_{12} in both cell lysis procedures using either buffer system. The residual 20% of radioactivity was distributed among the mitochondrial, polysomal and soluble fractions. The distribution of vitamin B_{12} after discontinuous sucrose density-gradient centrifugation of the 4000 *g* pellets is shown in Table 1. A major difference was observed between the two cell lysis methods. When a mortar and pestle was used, most of the labelled vitamin was found in the pellet, while in the case of ultrasonication the labelled vitamin was found in the chloroplast fractions. This indicated that the fraction binding vitamin B_{12} may vary in sedimentation coefficient depending on the method used to break the cells. To confirm this possibility, the final pellet obtained by sucrose density-gradient centrifugation of an extract prepared using a mortar and Tris buffer (this fraction contained cuticles, paramylon granules, nuclei and 96·5% of the labelled vitamin; Table 1) was homogenized together with the chloroplast fraction obtained by the same procedure. After centrifugation, the combined fractions were treated with ultrasound in glutamate buffer and the distribution of labelled vitamin was determined on a discontinuous sucrose density gradient. Such sonic treatment shifted 44% of the radioactivity to the 1·0–1·5 M-sucrose interface and 14% to the 1·5–2·0 M interface. This treatment resulted in fragmentation of nuclei but did not affect paramylon granules, which were still found intact in the pellet fraction. This result demonstrates that the cell fraction responsible for vitamin B_{12} binding can change from one sucrose density band to another according to the procedure used to lyse the cells.

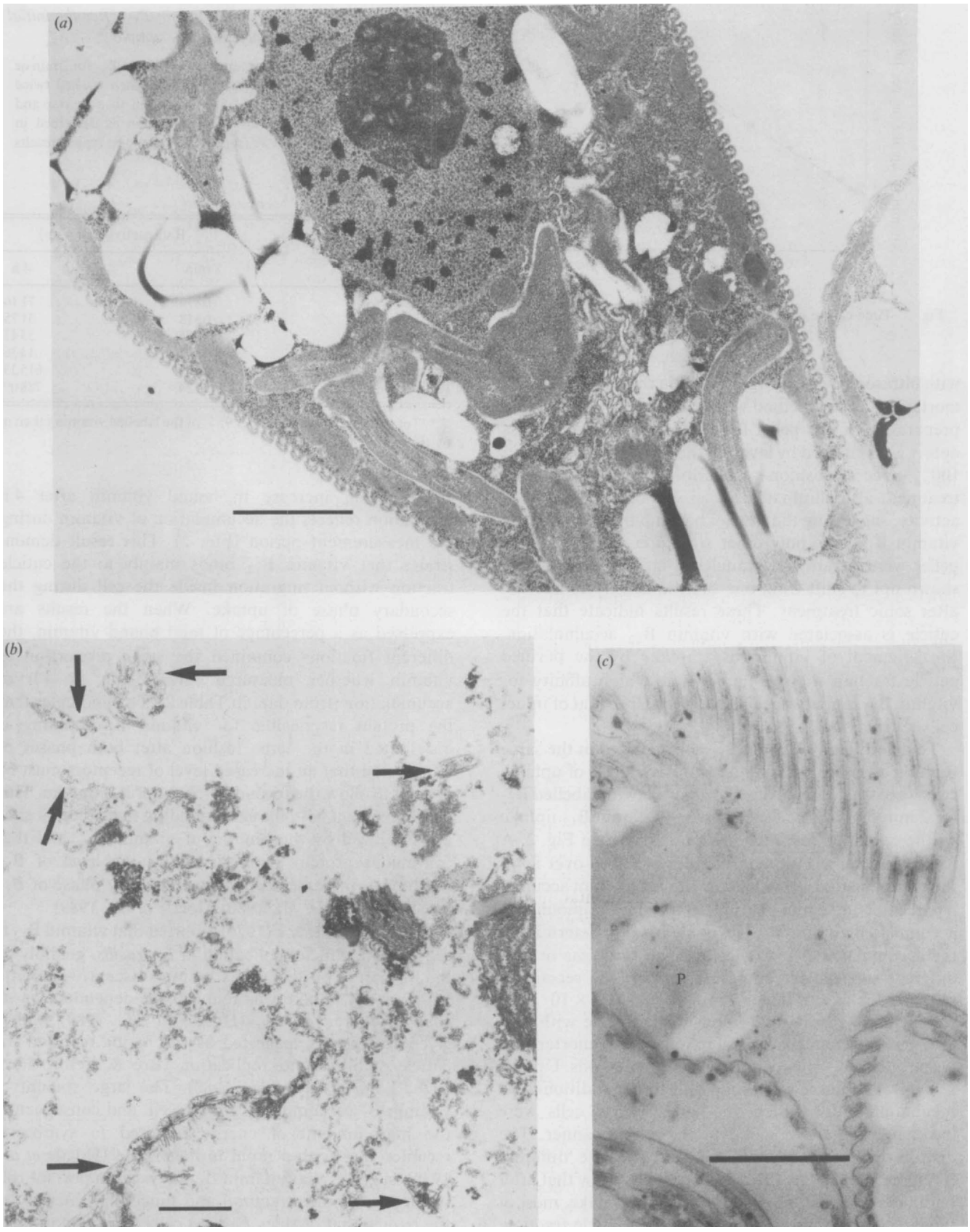
Table 1. *Vitamin B_{12} distribution in different sucrose gradient fractions after cell breakage by two methods*

Cells were labelled with [^{57}Co]B_{12} for 2 min in glutamate buffer (pH 3·5), washed and suspended in the appropriate buffer. Cells were broken either by ultrasonication or with a mortar and pestle, and then pelleted by centrifugation at 4000 *g* for 10 min. The pellet, suspended in the appropriate buffer, was layered on a stepped sucrose density gradient and centrifuged at 72000 *g* for 45 min. Each interface was then withdrawn, and a sample was used for radioactivity determination or for identification by light microscopy. Each value represents the mean of triplicate determinations from three separate experiments. The SE for each value did not exceed 10% of the mean. Superscripts: *a*, Broken chloroplasts and debris; *b*, intact chloroplasts; *c*, paramylon and broken nuclei (and broken glass for the mortar and pestle method); *d*, paramylon, cuticle, nuclei and broken glass.

	Vitamin B_{12} distribution (% of total)			
	Ultrasonication method		Mortar and pestle method	
Fraction	Glutamate (pH 3·5)	Tris (pH 7·5)	Glutamate (pH 3·5)	Tris (pH 7·5)
0·5–1·0 M	2·3	0·9	0·8	0·7
1·0–1·5 M	72·2[a]	29[a]	2·7[a]	1·2[b]
1·5–2·0 M	16·3[a]	68[a]	21·4[a]	1·1[b]
Pellet	8·5[c]	2·0[c]	75·5[c]	96·5[d]

Light microscopic identification of different cellular fractions is also shown in Table 1. No cell fraction was present at the 0·5–1·0 M-sucrose interface. With both cell lysis methods, intact or broken chloroplasts were always found at the 1·0–1·5 and 1·5–2·0 M-sucrose interfaces, and paramylon and nuclei (intact or broken) sedimented to the bottom of the tube. On the other hand, the cuticles were found in the pellet when cells were homogenized with a mortar, while these structures could not be found by light microscopy when ultrasonication was used. Fractionation by the latter technique probably sheared the cell cuticle into fragments that were too small for identification by light microscopy. We thus used electron microscopy to identify cuticle fragments in the chloroplast fraction obtained by the ultrasonic method at pH 3·5 (1·0–1·5 M-sucrose interface, containing 72% of the bound vitamin B_{12}; see Table 1). The typical shape of the cell cuticle is shown in Fig. 1 (*a*). This structure was found in the chloroplast fraction when cells were broken

Fig. 1. Electron micrographs of an intact cell and of the two cell fractions containing high amounts of bound vitamin B_{12}. (*a*) Intact cell. Note the structure of the cuticle. Bar, 1 μm. (*b*) Broken chloroplast fraction obtained when cells were broken by ultrasonication (1·0–1·5 M-sucrose interface in glutamate buffer). Note that several broken cuticle fragments are present within the chloroplast fraction (arrows). Bar, 1 μm. (*c*) Pellet fraction obtained using the mortar and pestle method in Tris buffer (pH 7·5). Note the high concentration of cuticle fragments. P, paramylon granule. Bar, 1 μm.


(a)
(b)
(c)
P

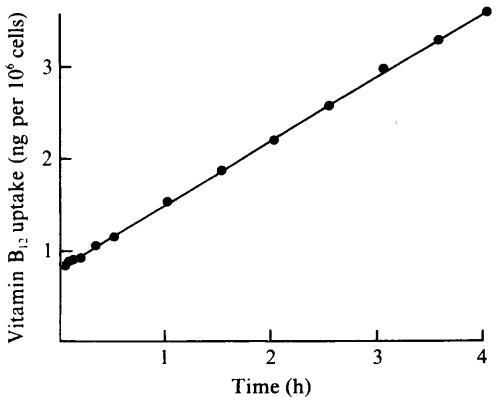


Fig. 2. Time course of vitamin B_{12} uptake by *Euglena gracilis*.

with ultrasonic waves (Fig. 1*b*) and in the pellet when the mortar and pestle method was used (Fig. 1*c*). During the preparation of this pellet for electron microscopy, glass debris was removed by layering the suspended pellet on a 100% glycerol cushion as decribed in Methods. This treatment also eliminated nuclei without loss of radioactivity, suggesting that nuclei have no binding sites for vitamin B_{12}. The only other structures present in this pellet were paramylon granules (Fig. 1*c*), which were shown not to shift from one sucrose density to another after sonic treatment. These results indicate that the cuticle is associated with vitamin B_{12} accumulation. Measurement of vitamin B_{12} uptake by the purified cuticle fraction showed that it had a high affinity for vitamin B_{12} (unpublished results) similar to that of intact cells (Sarhan *et al.*, 1980).

To determine if vitamin B_{12} accumulated in the same fraction after the initial and secondary phase of uptake, cells were fractionated after incubation with labelled B_{12} for 2 min or 4 h. The time course of vitamin B_{12} uptake by the cells used for fractionation is shown in Fig. 2. A fourfold increase in bound vitamin occurred over a 4 h incubation period as compared with the amount accumulated after a 2 min period. This high amount of accumulated vitamin made it convenient to determine if the accumulated B_{12} was localized in the same or in a different subcellular compartment after the secondary phase of uptake. After a 2 min period, $2{\cdot}5 \times 10^7$ cells were centrifuged at 4000 *g* and washed twice with cold Tris buffer. The cells were broken with a mortar and pestle and fractionated as described in Methods. Uptake was followed in the remaining cells for an additional 4 h incubation, after which another $2{\cdot}5 \times 10^7$ cells were harvested and fractionated in the same manner. The distribution of labelled vitamin B_{12} in the different fractions is shown in Table 2. The results show that after both the initial and secondary phases of uptake, most of the labelled vitamin was present in the cuticle fraction.

Table 2. *Distribution of bound vitamin B_{12} after the initial and secondary phase of uptake*

The same number of cells was labelled with [^{57}Co]B_{12} for 2 min or 4 h in glutamate buffer (pH 3·5). Cells were then washed twice with ice-cold Tris buffer (pH 7·5) and broken in a mortar and pestle with glass beads prior to fractionation as described in Methods. The experiment was repeated three times; typical results are presented.

	Radioactivity (d.p.m.)	
Fraction	2 min	4 h
Soluble	16874	71162
Ribosomes	6918	31756
Mitochondria	15805	55475
Chloroplasts	3567	14362
Cuticles	153560	615337
Total counts*	196724	788092

* 'Total counts' represents over 95% of the labelled vitamin taken up by the cells.

The fourfold increase in bound vitamin after 4 h incubation reflects the accumulation of vitamin during the measurement period (Fig. 2). This result demonstrates that vitamin B_{12} binds mainly to the cuticle fraction without migration inside the cell during the secondary phase of uptake. When the results are expressed as a percentage of total bound vitamin, the different fractions contained the same proportion of vitamin whether measured after 2 min or 4 h of accumulation (from data in Table 2). This indicates that the protein responsible for vitamin B_{12} binding is distributed in the same fashion after both phases of uptake and that an increased level of receptors must be present to allow the secondary phase of B_{12} uptake. This is in agreement with the earlier finding that receptor sites are stabilized by the binding of vitamin B_{12} and that continuous protein synthesis allows the level of B_{12} receptor to increase during the secondary phase of B_{12} uptake (Sarhan *et al.*, 1980; Houde *et al.*, 1989).

Hoffmann & Bouck (1976) reported that vitamin B_{12} is required for cuticle replication in *E. gracilis*, possibly by the formation of new crests by intussusceptive growth. Vitamin B_{12} uptake was found to be dependent on *de novo* protein synthesis (Houde *et al.*, 1989) and a glycoprotein was suggested as the factor required for cuticle stability and replication (Bré & Lefort-Tran, 1978; Lefort-Tran *et al.*, 1980). The large amount of vitamin B_{12} accumulated by the cell, and consequently the high amount of energy required to synthesize receptor sites with a rapid turnover rate (Houde *et al.*, 1989), suggests that vitamin B_{12} plays an important role in preserving the structural and functional integrity of the cuticle that enables *Euglena* cells to grow normally.

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