ASSIMILATION OF INORGANIC COBALT INTO RUMEN MICROORGANISMS

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SUMMARY

Assimilation of ionic cobalt by axenic cultures of four species of anaerobic rumen bacteria was measured using ^{60}Co as cobalt (II) chloride. Uptake could be measured in all four species but may not represent active accumulation in the case of *Lactobacillus casei* and *Propionibacterium acnes*. Significant uptake (89.5% of ^{60}Co present) was demonstrated by *Selenomonas ruminantium* from media containing nanomolar concentrations of inorganic cobalt and it was present within the cell as an organic cobalt complex in both lag and log phases of growth.

INTRODUCTION

Cobalt is required by ruminants for the synthesis of vitamin B_{12}. The latter is an obligatory requirement both for the mammalian host as well as for some ruminal microorganisms. The biosynthesis of cobalamin involves a complex series of reactions and must take place under anaerobic conditions. Microbial biosynthesis is thus the sole source of the vitamin which must be supplied to the mammalian host tissues as an organic (cobalamin) complex not as the element itself. Vitamin B_{12} is important in microorganism metabolism, where among other roles it is crucial to the formation of propionate as a major energy source and the major precursor for hepatic gluconeogenesis. (Macrae and Lobley 1986). The ruminants' major metabolic transformations which require cobalt (coenzyme B_{12}) occur in the liver. Mammalian metabolism of propionate utilizes methyl malonyl CoA isomerase, a coenzyme B_{12}-requiring enzyme to catalyze the conversion of methyl malonyl CoA to succinyl CoA. (Beck et al. 1957), and it is here that conversion of propionate to glucose is inhibited when cobalamin levels are limiting..

The physical manifestation of cobalt deficiency in the ruminant is one of gradual loss of appetite and lack of growth or loss of body weight followed by extreme inappetence, wasting and anemia then death. (Underwood 1977). Biochemical changes accompanying these physiological changes include a decline in cobalt concentration in liver and kidney, low plasma levels of - glucose, alkaline phosphatase, ascorbic acid and thiamine, and elevated levels of glutamate oxaloacetate transaminase and pyruvate. (MacPherson et al. 1976) In addition blood propionate and acetate clearance times are increased (Somers et al. 1969) in association with an increase in plasma methyl malonic acid (Rice et al. 1989). The muscle wasting and subsequent death in

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extreme deficiency states, is due solely to the depressed feed intake caused by the inappetence brought on by the retardation of the rate of propionate metabolism (Marston et al. 1972) and the subsequent rise in plasma methyl malonic acid, (Rice et al. 1989).

Two factors may contribute to ruminants' susceptibility to cobalt deficiency. The first is the inefficient conversion of cobalt to vitamin B_{12} by rumen microorganisms. Smith and Marston (1970)) showed that under cobalt deficiency conditions about 15% of dietary cobalt was converted to cobalamin in the rumen whereas only 3% was converted when cobalt was at an adequate level in the diet. Gawthorne (1970) showed that 44% of the vitamin B_{12} activity (i.e. B_{12} plus analogues) in sheep fed a cobalt sufficient diet (0.34ppm) was the physiologically inactive form, 2 - methyladenyl cobamide. This proportion remained the same even when the dietary cobalt was dropped to 0.04ppm however the proportion of the physiologically active 5, 6 - dimethyl benzimidazolyl cobamide produced in this deficient cobalt state, increased from 35 to 63% at the expense of other vitamin B12 analogues. Therefore a greater proportion of the "vitamin B12" synthesized by rumen microorganisms growing under cobalt deficient conditions was in a form that could be utilized by the host animal, however, the total quantity produced was far less than that produced when on a cobalt sufficient diet.

The second factor possibly contributing to the ruminant's susceptibility to cobalt deficiency is the degradation and poor absorption of vitamin B_{12} produced in the rumen. As low as 3% has been estimated to be absorbed. (Kercher and Smith 1955.)

To date no evidence has been found to show that cobalamin is either produced or required by plants, therefore the only source of supply to ruminants is via synthesis by rumen microorganisms; their supply of cobalt being provided by that contained in pasture and cereals in the rumen digesta, the cobalt content of which is very variable, although normally small. Cobalt is usually transported in plants as either a low molecular weight (1000 - 5000 daltons) organic complex or as the cation. (Aller et al. 1990).

In order to better understand the factors affecting the utilization of plant derived cobalt this study looked at the uptake of ionic cobalt by various rumen bacteria.

MATERIALS AND METHODS

Bacterial species

The microorganisms used in this study were isolated and characterized by Dr. Frank Hudman. Axenic cultures of four rumen bacterial species were investigated: ***Butyrivibrio fibrisolvens*** and ***Lactobacillus casei*** subspecies casei, (both expected to exhibit a low requirement for cobalamin) also ***Propionibacterium acnes*** serotype 2, and ***Selenomonas ruminantium*** (chosen for an expected elevated requirement for cobalamin based on their production of propionate as a major fermentation product). Cultures were stored frozen in medium containing 20% glycerol according to the method of Teather (1982). In all experiments the

maintenance of species purity was checked by Gram stain.

Media

The liquid culture medium was a modification of either Caldwell and Bryant's (1966) medium IO or Bryant and Burkey's (1953) rumen fluid (RF) medium as set out in Table I. All chemicals were AR grade unless otherwise indicated. Volatile fatty acids (VFA) stock solution was prepared by adjusting (with sodium hydroxide to pH 7.0) a solution containing the following fatty acids (in the volumes indicated in mL) acetic acid 17.0, propionic acid 6.0, butyric acid 4.0 and isobutyric acid, valeric acid, isovaleric acid and dl a-methylbutyric acid 1.0 each, to pH 7.0 and diluting to 1.0 L. Mineral solution #I contained K_2HPO_4 at 3.0 g/L. Mineral solution #2 consisted of the following components at the concentrations given (in g/L) KH_2PO_4, 3.0; $(NH_4)_2SO_4$ 3.0; NaCl, 6.0; $CaCl_2.2H_2O$, 0.3; and $MgSO_4.7H_2O$ 0.3. "Mineral mix" consisted of (g/l) KH_2PO_4, 9.0; K_2HPO_4, 9.0; NaCl, 9.0; KCl, 9.0; $CaCl_2.2H_2O$, 0.2; $MgCl_2.6H_2O$, 0.2; $MnCl_2.4H_2O$, 0.1; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1 and Na_2SO_4, 0.285.

TABLE 1 Media Composition

Component	Percent in medium	
	Modified medium 10	RF medium
Clarified rumen fluid	-	33.0
Glucose	0.1	0.2
Cellobiose (Sigma)	0.1	0.2
Tryptone (Difco)	0.1	
Peptone (Difco)	-	0.1
Yeast Extract (Difco)	0.05	0.1
Sodium carbonate	0.4	
Sodium bicarbonate	-	0.5
Mineral solution #1	-	16.5
Mineral solution #2		16.5
VFA stock solution	1.0	1.0
Mineral mix	5.0	
Resazurin (BDH)	0.0001	0.0001
Hemin (Sigma)	0.0001	-
Cysteine HCL (Sigma)	0.02	0.02

Media was deoxygenated by boiling under a stream of CO_2/H_2 gas (95:5) which was passed through a column of hot (400°C) reduced copper turnings to remove traces of oxygen. Cysteine HCl was added to the cooling medium and when cool was dispensed by methods described by Miller and Wolin (1974) into Hungate tubes or serum bottles. Which after sealing were autoclaved at 107° C for 45 minutes. Modified medium 10 was used for all uptake studies whereas RF medium was used for storing cultures at -20°C. The anaerobic procedures used were based on those of Hungate (1950), Bryant and Burkey (1953) and Miller and Wolin (1974).

Transfers of inoculum were carried out by the methods of Macy et al. (1972) using sterile disposable syringes. All manipulations of cultures were carried out either in an anaerobic hood (Kaltec Pty Ltd, 23 Bennet Rd, Melrose Park, South Australia) or in a bench mounted glove box (AtmosBag, Aldrich Chemical Co), following procedures outlined by Aranki and Fretor (1972).

Cobalt uptake

(i) Frozen cultures from storage were grown as an overnight culture This was then stored at 4oC and could be used as the initial inoculant for any number of cultures for a period of weeks. When an inoculum for an uptake experiment was required the culture at 4°C was subcultured (1% v/v for 6 hours for *S.ruminantium* and 2% v/v for 15 hours for other species). This was again subcultured *(1%* v/v for S. ***ruminantium*** for 5 hours and 2% v/v for IO hours for other species) and it was this culture that was used as the inoculum for each of the uptake experiments.

(ii) Measurement of uptake Media for the uptake experiments were stored in an incubator at 39°C for several hours for temperature equilibration prior to inoculation. Cobalt-60 (92.5 kBq as Cobalt (II) chloride in 0.1M HCl, Amersham Australia Pty Ltd) in 2.5 mL modified medium IO was added to each of three 150 mL serum bottles containing 140 mL modified medium IO and (as controls) 2.5 mL modified medium IO (containing no 60 Co) was added to each of another three 140 mL medium. Each was then inoculated using the prepared inoculum (1% v/v for ***S. ruminantium*** and 5% v/v for other species) and returned to the incubator.

At time intervals appropriate to the growth rate of each species 20 ml aliquots were taken from each of the 6 cultures as well as two ml to monitor growth (at 650 nm). A quantity of medium containing ^{60}Co equivalent to that in 20 ml of the test cultures was added to the three control cultures. The three were inverted several times and all six immediately centrifuged at 20,800 g for 15 minutes. The bacterial pellet was washed once with medium (no ^{60}Co) and then once with 0.9 % saline after which it was sedimented through silicone oil (an II to 40 mixture of Dow Corning Silicone oils, types 200 and 550 respectively). The s.g. of the oil mixture was intermediate between that of the bacteria and the aqueous medium. After removal of the oil, the bacterial pellet was stored at -20°C prior to assaying the gamma activity in a Packard Modumatic VI Auto-gamma counter. Protein determination was also carried out on the pellet by the biuret reaction (Herbert et al.1971).

Gel chromatography

Several cultures were prepared by inoculating (as previously described) medium containing 74 kBq of ^{60}Co in 80 mL of medium. After 2 h. growth at 39°C, cells were harvested, washed twice in growth medium, pelleted through silicone oil (as before), and lysed by sonication in 1.0 mL of $CoCl_2$ solution (I mg/mL). The procedure was repeated with a 5 h. culture (end of log phase).

The lysates were subjected to Sephadex G-IO chromatography under conditions which clearly separated co++ and cobalamin.

Radio-labelled cobalt was added to lysate from a parallel culture grown in the absence of ^{60}Co to test for the occurrence of any spontaneous complex formation between Co^{++} ions and cellular components.

RESULTS AND DISCUSSION

Cobalt uptake for the four species is shown in Fig. I

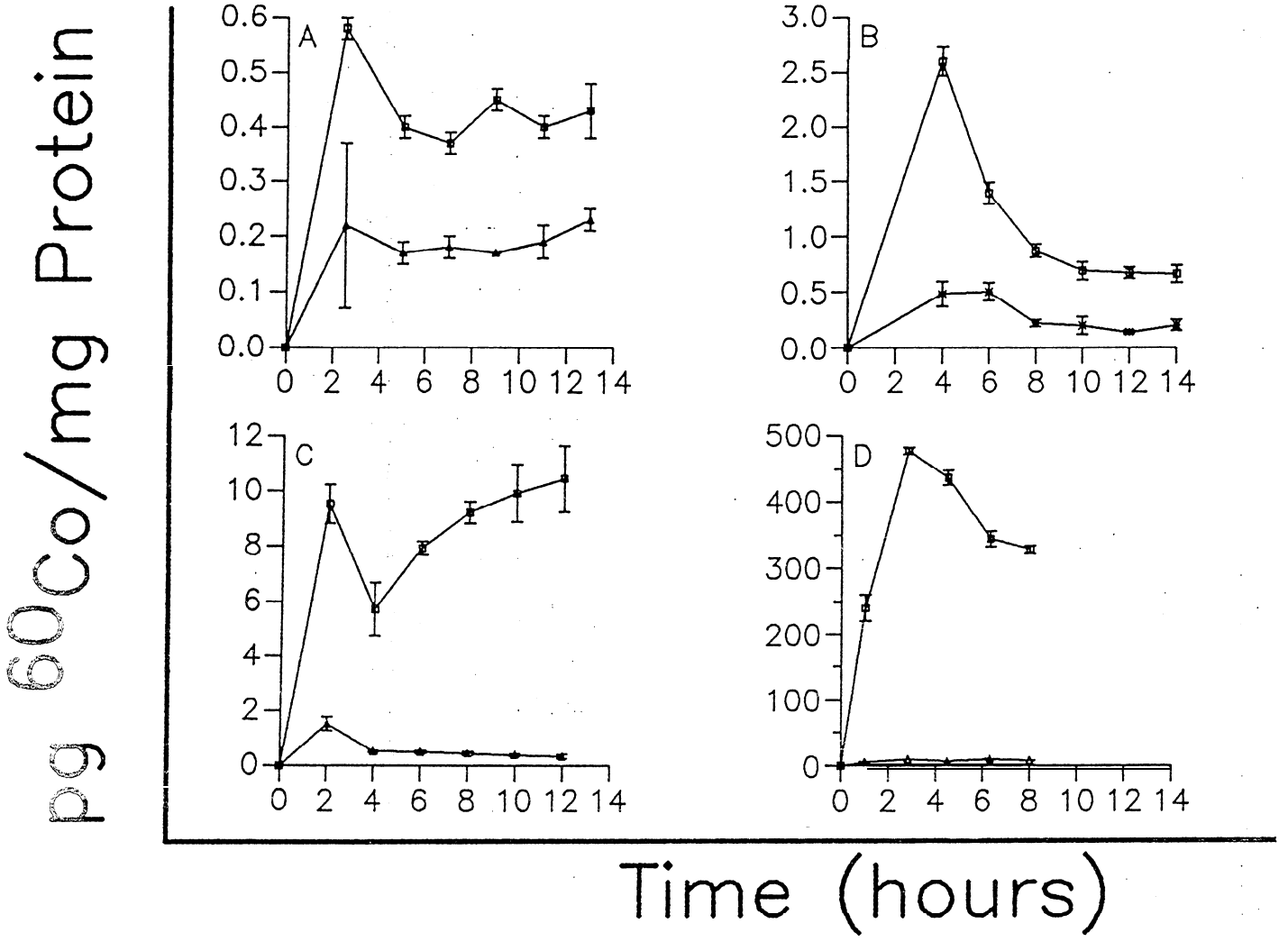

Figure 1. UPTAKE OF IONIC COBALT-60 DURING LOGARITHMIC GROWTH PHASE OF FOUR AXENIC CULTURES OF RUMEN BACTERIA.

Uptakes are presented as the mean of three determinations and their standard deviations. Measurements were made as described under "Methods". Control cells ( ) at the identical stage of growth as the test cells were exposed to the isotope for a brief period (ca. 1 min.) before separation of the cells from the medium. Test cells ( ) were exposed to the isotope for the time indicated on the abscissa.

A. ***Propionibacterium acnes*** (serotype 2)
B. ***Lactobacillus casei*** (subspecies casei)
C. Butyrivibrio fibrisolvens
D. ***Selenomonas ruminantium***

Note the different scale on the ordinate in each frame.

From the Fig. it can be seen that the total quantity of cobalt taken up by L. casei is about equal to that taken up by P. acnes. After the initial peak in lag phase the quantity taken up per mg protein is also of the same order of magnitude.

Examination of figure 2. shows that ionic cobalt and high molecular weight complexes of the metal are readily distinguishable after chromatography on a Sephadex G-IO column (300 mm x IO mm). Part (D) of this figure also indicates that incorporation into high molecular weight fractions is not due to artefactual association of cobalt ions with high molecular weight fractions of the cell. Parts (B) and (C) of figure 2 show that cell contents from lysed *S. ruminantium,* at different stages of growth, retain a considerable fraction of the absorbed cobalt as a high molecular weight form.

Dryden et al. (1962) have shown that whereas S. ruminantium and B. fibrisolvens "gave evidence of appreciable synthesis" of vitamin B_{12} or its analogues, P. acnes and an anaerobic Lactobacillus did not. P. acnes and S.ruminantium use the same metabolic pathway for propionate production (Schlegel 1986) and certain species of ***Lactobacillus*** require an adenosyl cobalamin dependent ribonucleotide reductase (Sennett et al. 1981). It is possible therefore that all four species require cobalamin or an analogue and must transport either preformed cobalamin or synthesize it from ionic cobalt within the cell. If P. acnes and *L. casei* do not synthesize cobalamin ***de novo*** then they probably possess uptake mechanisms for the accumulation and active transport of preformed cobalamin across the cell membrane and the small amount of uptake of ionic cobalt seen here must represent equilibration of the cobalt concentration between the medium and cytoplasm.

TABLE 2 PERCENTAGE OF COBALT-60 ASSIMILATED

Species	%
P. acnes	0.044
L. casei	0.033
B. fibrisolvens	2.13
S. ruminantium	89.5

The figures given above are expressed as the percentage of cobalt-60, added to the growth medium, which was recovered in the bacteria. The time required for the incorporations reported above varied with the species, as follows: ***P. acnes*** 13 h; ***L. casei*** 14 h; ***B. fibrisolvens*** 12 h; ***S. ruminantium*** 8 h.

If both ***S. ruminantium*** and ***B. fibrisolvens*** have the ability to synthesize cobalamin or analogues for their own metabolic needs, from Fig. 1 and Table 2 the rate and quantity of ionic cobalt assimilation by ***S. ruminantium*** would indicate production of macromolecular cobalt far in excess of its own requirements. The extent of this uptake will be revealed by kinetic studies.

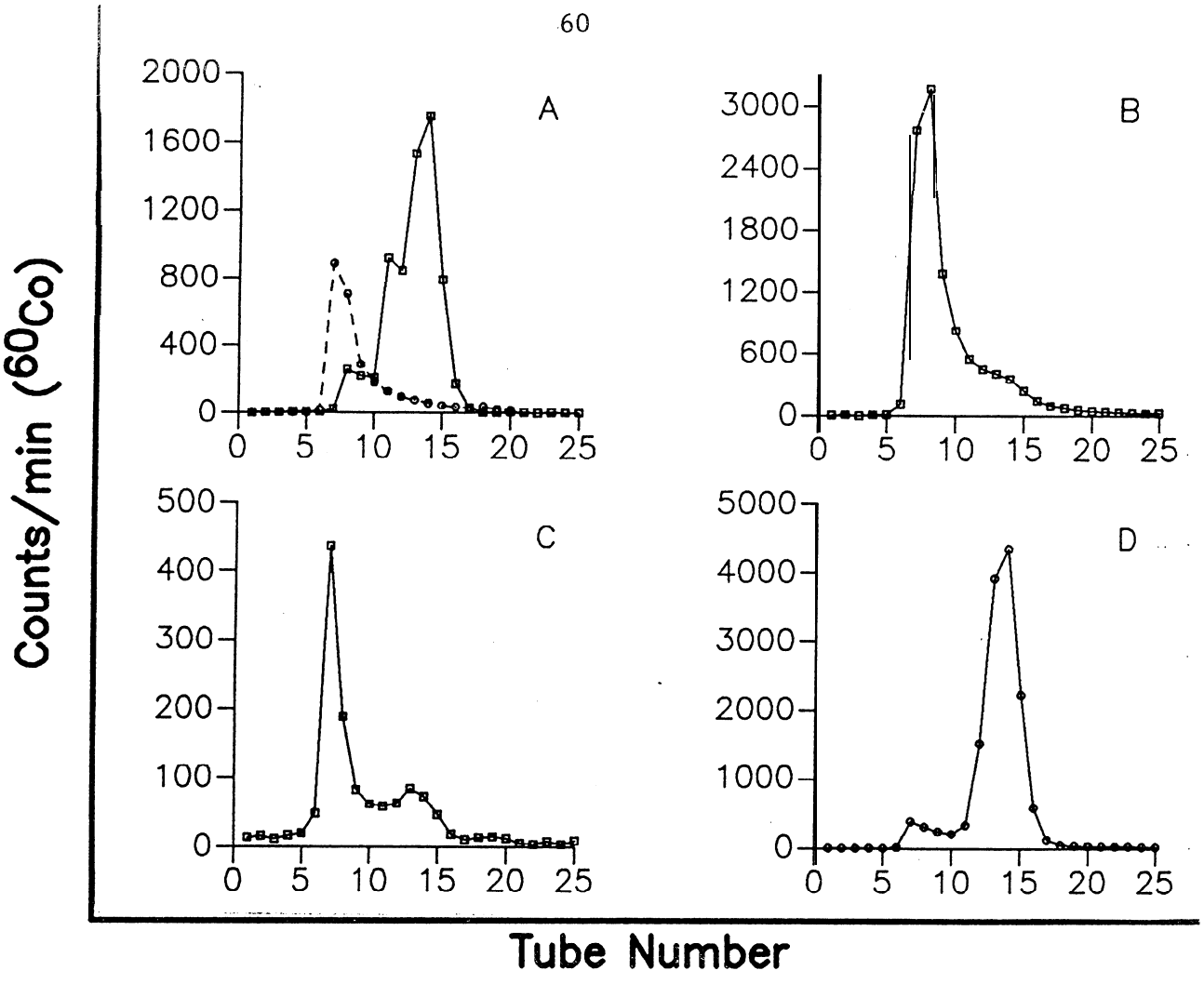

Figure 2. INCORPORATION OF INTRACELLULAR COBALT INTO HIGH MOLECULAR WEIGHT FRACTIONS.

The four frames above are representative elution profiles from gel filtration chromatography on Sephadex G-10 under conditions designed to separate Co^{++} from high molecular weight complexes. The dashed line in, frame (A) indicates ultraviolet light absorption due to non-radioactive vitamin B_{12} and this frame also shows the separation of an artificial mixture of cobalt-60 ions and the non-radioactive vitamin. Frame (B) shows the separation of cell contents from late log phase cells, whilst (C) is a similar experiment with early log phase cells. Frame (D) shows a separation of unlabelled cell contents to which cobalt-60 was added after cell lysis.

Estimation of the adventitious cobalt levels present in the bacterial growth medium were carried out by atomic absorption spectroscopy and found to be in the range $50\text{-}100 \times 10^{-9}$ molar. Thus it is clear that ***S. ruminantium*** can remove approximately 90% of the cobalt from this very dilute solution and convert it to the complexed form (presumably cobalamin). The organism thus contains a very high affinity transport system for cobalt, and the specificity of this system is presently under investigation. Since all of the cobalamin for the ruminant animal,(and at least a substantial part of the vitamin B_{12} supplied in human diets) is synthesized by rumen microflora, it appears that the ***Selenomonas*** organism may constitute an important entry portal for cobalt moving from the geosphere to the biosphere.

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