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Tracer Studies with the B₁₂ Vitamins2. BIOSYNTHESIS OF VITAMIN B₁₂ LABELLED WITH ⁶⁰Co AND ³²P

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In 1949 we attempted to obtain radioactive vitamin B₁₂ from the livers of rats and pigs receiving ⁶⁰Co salts in their diets (Braude, Free, Page & Lester Smith, 1949; Cuthbertson, Free & Thornton, 1950). These attempts failed because the livers contained negligible amounts of vitamin B₁₂. We recognized at the time that it would have been preferable to use ruminants, but such animals were not available to us. Subsequent work suggests that we ought to have extracted the faeces rather than the livers of our animals.

About the same time we succeeded in preparing very small amounts of radioactive vitamin B₁₂ concentrates by fermentations with *Aerobacter aerogenes* isolated from hen faeces. More recently we have returned to this problem, using *Streptomyces griseus*; in the meantime the preparation by this method of [⁶⁰Co] vitamin B₁₂ with low or moderately high specific activity has been reported (Chalet, Rosenblum & Woodbury, 1950; Rosenblum & Woodbury, 1951).

RESULTS

Preparation of vitamin B₁₂ containing ⁶⁰Co

To avoid diminishing the specific activity of the added radioactive cobalt, we used a semi-synthetic medium (Dulaney, 1948) shown to be nearly free from cobalt. We added only about 0.2 p.p.m. of radio-cobalt, thus minimizing any radiation hazard and securing efficient conversion of cobalt to vitamin B₁₂. The fermentation liquors were worked up to yield a few mg. of crystalline vitamin B₁₂ by a simple procedure described in the Experimental section. The latest fermentation with ⁶⁰Co at 3.5 mc./mg.

has yielded vitamin B₁₂ with a specific activity of 120 μ c./mg., which is considerably higher than any previously recorded (Rosenblum & Woodbury, 1951).

Preparation of vitamin B₁₂ containing ³²P

It was of interest to attempt the preparation of vitamin B₁₂ labelled with radioactive phosphorus, since this would be safer for projected human metabolism experiments than vitamin B₁₂ containing radioactive cobalt with its much longer half-life and penetrating gamma radiation. It was foreseen that it would be difficult to prepare material with adequate specific activity because preliminary experiments had shown that *Streptomyces griseus* needs a moderately high level of phosphate in the medium, growth and vitamin B₁₂ production being impaired when the total phosphorus level is reduced to about 0.01 %.

It was decided to use 10 mc. of ³²P and to aim at preparing about 1 mg. of the radioactive vitamin. It was then almost immaterial what volume of medium was used for the fermentation; the smaller the volume, the higher the specific activity of the resulting vitamin B₁₂ would be, but the greater the proportion of ordinary vitamin B₁₂ that would have to be added.

The radioactive phosphate was added aseptically 24 hr. after fermentation started. This was done in the hope that part of the phosphorus in the medium would already have been taken up by the mould and converted into organic phosphorus compounds that would not exchange with the added ³²P. This hope was not realized; the specific activity of the product was the same as that from a pilot run with

the ^{32}P added before inoculation. The yield of crystalline vitamin B_{12} was 0.91 mg. with a specific activity of $0.35 \mu\text{c./mg.}$ Less than one part in 10 000 of the ^{32}P added was present in the vitamin B_{12} produced by this fermentation.

EXPERIMENTAL

Fermentations with ^{60}Co

Several fermentations have been carried out with ^{60}Co of the highest specific activity available at the time, namely:

Specific activity of ^{60}Co (mc./mg.)	Specific activity of resulting vitamin B_{12} ($\mu\text{c./mg.}$)
0.35	14
0.50	18.5
3.5	120

A synthetic medium (Dulaney, 1948) containing 0.2 p.p.m. of ^{60}Co was employed. The fermentations were carried out in bottles containing 3–15 l. of medium.

After autoclaving and inoculating with a vegetative culture of *S. griseus*, the bottles were fixed in a water bath thermostatically controlled at 27° and the contents were stirred and aerated for 4 days. The broths usually contained 0.5–1 $\mu\text{g.}$ of vitamin B_{12} /ml. assessed by microbiological assay with an *Escherichia coli* mutant (Harrison, Lees & Wood, 1951).

Extraction of vitamin B_{12} from fermentation liquors

The broths were processed in slightly different ways; the following procedure is the simplest. KCN (1 g.) was added to 2.5 l. of the harvested broth. The mixture was allowed to stand 1 hr. at pH 8 and then (for convenience) overnight at pH 5. It was then boiled for 5 min. and filtered with the aid of kieselguhr; the residue was washed with a little water.

Charcoal (30 g., Sutcliffe Speakman no. 5 as purchased) and 15 g. of kieselguhr were slurried with water and made into a bed on a 14 cm. Büchner funnel. One-third of the broth plus washings was run slowly through this bed, followed by 400 ml. of 6% phenol to elute impurities and permit adsorption of additional vitamin B_{12} . Two further similar portions of broth and 6% phenol were run through the same bed. The adsorbate was eluted *in situ* with 1.5 l. of 60% (v/v) aqueous acetone. This eluting agent gives a clean product that can be worked up with little additional loss, although it does not remove the whole of the activity from the charcoal. Continued elution with 300 ml. of 90% phenol brought off additional vitamin B_{12} equivalent to about one-quarter of that originally present in the broth.

The acetone eluate was concentrated under reduced pressure to 32 ml. after addition of a trace of cyanide. The concentrate was shaken with 40 ml. of butanol and 16 g. of

$(\text{NH}_4)_2\text{SO}_4$. Some black sludge was precipitated; it caused an emulsion, which was broken by filtering the whole mixture through kieselguhr. The sludge was redissolved in a little dilute NaHCO_3 solution and added to the aqueous layer, which was given two further extractions each with 50 ml. of butanol. The combined butanol extracts were shaken with successive small portions of water acidified to about pH 3 with HCl. Washing was continued until no further pink colour was removed.

The combined aqueous extracts were concentrated to 5 ml. and extracted twice with 1 vol. of phenol-benzene 1:2. The solvent extracts were washed with dilute NaHCO_3 and with water. The bulked solvent extracts were mixed with about 2 ml. of acetone and 40 ml. of ether. After 1 hr. the red precipitate was collected by centrifuging. It was redissolved in about 1 ml. of ethanol, centrifuged from a trace of insoluble matter and reprecipitated with ether. The precipitate was dissolved in a few drops of water and acetone was added; crystallization occurred almost immediately. The product, after recrystallization from aqueous acetone, was characterized as vitamin B_{12} by its absorption spectrum. Yields of crystalline vitamin B_{12} equivalent to 30–40% of that present in the broth have been obtained by this extraction procedure or similar ones.

Vitamin B_{12} containing ^{32}P

The medium (200 ml.) containing approximately 0.01% total P was distributed into five 250 ml. conical flasks. After autoclaving and inoculation with a vegetative culture of *Streptomyces griseus* the flasks were incubated at 27° on a rotatory shaker. At 24 hr. 2 mc. of ^{32}P in the form of sterile 'carrier-free' phosphate was added aseptically to each flask. Growth of mycelium was poor; subsequent experiments with lower levels of ^{32}P showed that this was not due to radiation damage but to the sub-optimal level of total P in the medium.

The broth was harvested at 4 days. It was adjusted to pH 4.5, heated in a boiling-water bath for 20 min., then filtered. The vitamin B_{12} titre was $0.5 \mu\text{g./ml.}$ KCN (0.1 g.) was added and 'carrier' vitamin B_{12} (1.8 mg. anhydrous). In view of this fortification, the charcoal step was omitted and the liquor was extracted directly with butanol in presence of $(\text{NH}_4)_2\text{SO}_4$ followed by the other step described above.

SUMMARY

1. ^{60}Co has been incorporated in vitamin B_{12} by fermentation with *Streptomyces griseus*.
2. The crystalline product had the very high specific activity of $120 \mu\text{c./mg.}$
3. ^{32}P has been similarly incorporated in vitamin B_{12} .
4. Competing metabolic requirements for phosphorus resulted in a low efficiency of conversion and a specific activity of only $0.35 \mu\text{c./mg.}$

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