Conversion of Cyanocobalamin to a Physiologically Occurring Form

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It is well known that the therapeutically most common form of vitamin B12, cyanocobalamin (CN-B12), does not normally occur in human tissues, where cyanide-free forms (OH-B12) dominate. The purpose of the present studies was to see whether cyanocobalamin is converted to a physiologically occurring form. In solution, cyanocobalamin is very stable, so that the mechanism of a possible conversion should be of interest. Similarly, it would be of interest to see whether clinical conditions exist where no conversion occurs. The first part of these studies, using the reverse isotope dilution technic in dogs, showed that CN-B12 is converted to a noncyano form, OH-B12, over a period of approximately 5 weeks after administration.

The aims of the investigation here reported were to confirm the original results, to simplify the technic, to study transformation of CN-B12 in species other than the dog, to localize the process, and to determine the rate of conversion.

Materials and Methods

Studies were carried out in guinea pigs (weight approx. 400 Gm; age 3 months) and on healthy human volunteers. Radio-CN-B12-Co57 (2 μc./μg.) and radio-OH-B12-Co57 (2 μc./μg.) from Merck, and radio-CN-B12-Co58 (90 μc./μg.) from Philips were used. Guinea pigs received 0.02 μg. CN-B12 subcutaneously twice daily for 3 to 90 days. Each of the volunteers received a single intramuscular injection of 200 μg. B12.

Guinea pig livers were removed immediately after sacrifice, then homogenized in physiologic saline (1 Gm./5 ml.) at 4 C. with an EHMEDA homogenizer (60 minutes at 10,000 rpm), and centrifuged (5 minutes at 900 g). Sediments were discarded. Any livers or homogenates kept in storage were frozen. In the human subjects urine was collected for 12 hours after CN-B12 administration.

For liberation of B12 from protein binders, one or more of the following procedures were employed (the parenthesized abbreviations are for identification, in Fig. 1. of the combinations used): Pepsin digestion3 (P); boiling for 30 minutes (B); autoclaving, 118 C., 1.2 Kg./sq.cm., 120 minutes (A); treatment with 0.1 mg. KCN/Gm. liver (CN); papain digestion 150 mg. papayotin (Merck "1:350") per gram liver, 56 C., 2 hours (Pa). The degree of B12 liberation was determined by exhaustive dialysis. Following liberation with papain alone, 80 per cent of the radioactivity was dialyzable.

Extraction. B12 was extracted from 6 ml. homogenate supernatants with 0.6 ml. cresol-carbonetetrachloride (meta-cresol 0.3 ml., CCl4 0.3 ml.). The cresol phase was

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washed with 0.3 ml. CCl₄ and 0.6 ml. water, whereafter 0.48 ml. sec. butanol, 0.48 ml. CCl₄, and 0.3 ml. water were added. Before the cresol phase was discarded, it was washed twice with 0.3 ml. water. The pH was adjusted to 5.0 with an acetate buffer. With this procedure, some 80 per cent was recovered of radioactive CN-B12 or of radioactive OH-B₁₂ added to liver homogenates.

**Incubation** was done in a water bath at 37 C. Toluene was occasionally used for bacteriostasis but did not appreciably influence the results.

**Paper chromatography** proceeded for 19 hours at 37 C. on Whatman no. 1 paper, using sec. butanol saturated with 3 per cent acetic acid. Twenty cu. mm. extract was applied. The paper was cut into 1 cm. strips, which were measured in a well-type scintillation detector for the time required to give a standard deviation of the counts of less than 5 per cent. Therapeutically used pharmaceutical CN-B₁₂ ("Cycobemin," ACO) and OH-B₁₂ ("Oxobemin," Vitrum) were used as standards and run on each chromatography strip. Before application to chromatography paper, extracts from human urine were mixed with standards in order to preclude influence of salts in the urine and bacteriostatics in the standard.

Final results were controlled as follows. When the radioactivity in liver extract had been identified with nonradioactive standards—e.g., CN-B₁₂—a new aliquot of the same liver extract was chromatographed and pure radioactive CN-B₁₂ applied in the same spot on the paper as the liver extract. If a single chromatographic radioactivity peak resulted, the liver radioactivity was considered to be chromatographically equivalent with CN-B₁₂.

Since the solvent front left the paper, values were expressed relative to CN-B₁₂ standard (R). In the following, relative amounts of OH-B₁₂ and CN-B₁₂ refer to the amounts found on the chromatography paper.

**RESULTS**

**Stability and Errors.** The standard deviation of hydroxocobalamin Rᵣᵣᵣᵣ in 41 standard samples, run in 17 different assays, was 0.07. The mean OH-B₁₂ Rᵣᵣᵣᵣ was 0.32. Paired standards run in the same assay had a standard deviation of 0.057 (intraclass variance). Different brands of pharmaceutical CN-B₁₂ varied remarkably in behavior. Cycobemin migrated, on the average, 9 per cent faster than Hepagon (Astra); "Alpha redisol" (Merck, Sharpe & Dohme), 34 per cent faster than Oxobemin.

CN-B₁₂-Co₅⁷ stored for one month in a position exposed to sunlight had a major peak between OH-B₁₂ and CN-B₁₂ (Rᵣᵣᵣᵣ 0.45–0.87) probably indicative of breakdown. No sign of decomposition was noted in dark-stored frozen samples.

**Recovery of Liberated B₁₂.** With each of the aforementioned liberation methods, most of the B₁₂ added in vitro to liver homogenate was dialyzable. Both OH-B₁₂ and CN-B₁₂ thus liberated were, however, chromatographically less mobile than the corresponding standard (Fig. 1). Similar behavior was noted for radio-B₁₂ administered in vivo (parenterally), when studied in liver homogenate.

**Recovery of Extracted B₁₂.** Following in vitro addition to liver homogenate, and after liberation and extraction, the radioactivity was chromatographically identical to CN-B₁₂ when radio-CN-B₁₂ had been added, and to OH-B₁₂ when radio-OH-B₁₂ had been added.

**Conversion in Vitro.** Following 24 hours incubation (Table 1) at a B₁₂ concentration approximating the physiologic level in the liver, the radioactivity
added as CN-B₁₂ had in great part undergone transformation to OH-B₁₂. After incubation for only 2 hours, on the contrary, no conversion was demonstrable. Nor was any transformation detectable in boiled liver homogenate. These negative results are not shown in the table. Papain liberation of B₁₂ prior to incubation tended to facilitate conversion.
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Table 1.—In Vitro Conversion of CN-B₁₂ to OH-B₁₂ by Liver Homogenate*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Mean</th>
<th>S.E. of the Mean</th>
<th>Treatment of Liver Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>13, 14, 15</td>
<td>9</td>
<td>3</td>
<td>Papain† digested after incubation. Incubated 24 hours at 37 C.</td>
</tr>
<tr>
<td>21, 22, 24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33, 37, 38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40, 41, 42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28, 29, 46</td>
<td>73</td>
<td>27</td>
<td>Papain† digested before incubation. Incubated 24 hours at 37 C.</td>
</tr>
</tbody>
</table>

*0.56 mg. radio-CN-B₁₂ were added in vitro per gram of liver.
†150 mg. papain/g liver, 2 hours. 56° C.

Table 2.—In Vivo Conversion of CN-B₁₂ to OH-B₁₂ in Guinea Pigs

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Time Between Final Injection of CN-B₁₂ and Sacrifice</th>
<th>Fraction of Radioactivity Found in OH-B₁₂ Peak</th>
<th>Mean</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 A, 3 B,</td>
<td>30 days</td>
<td></td>
<td>93</td>
<td>5</td>
</tr>
<tr>
<td>6 A, 10 B,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27, 39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51, 54</td>
<td>15 days</td>
<td></td>
<td>70</td>
<td>—</td>
</tr>
<tr>
<td>50</td>
<td>10 days</td>
<td></td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>35, 48, 49</td>
<td>5 days</td>
<td></td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

The differences between the means at different times are not statistically significant but secondary to the variation between individual animals.

Conversion in Vivo. In guinea pigs which received radio-CN-B₁₂ parenterally for 3 months and were sacrificed one month after the final dose, radioactivity generally was recovered as OH-B₁₂ (Fig. 2). In two of seven experiments, however, one-third of the radioactivity persisted as CN-B₁₂.

Similarly, for the animals that were injected with radio-CN-B₁₂ for 3 days and then sacrificed 5, 10, and 15 days, respectively, after the final injection, the radioactivity derived as a rule from OH-B₁₂; in the two 15-day animals one-third of it was still CN-B₁₂ (Table 2).

Reconversion of OH-B₁₂ to CN-B₁₂. In order to check the identity of the radioactivity which was isolated after parenteral CN-B₁₂ administration and which behaved chromatographically as OH-B₁₂, potassium cyanide was added prior to chromatography. As a result, the OH-B₁₂ was invariably reconverted back to CN-B₁₂ (Fig. 3).

Recovery of Radio-B₁₂ Extracted from Human Urine. Following in vitro addition of radioactive OH-B₁₂ or CN-B₁₂ to human urine, incubation for 20 hours at pH 7 and 37 C., most of the radioactivity was dialyzable and was recovered in the form in which it had been added. After parenteral CN-B₁₂ administration too, urinary radioactivity was still CN-B₁₂.

Rate of Conversion in Guinea Pigs. For animals which had received 39 µg.
Fig. 2.—Paper chromatography of radio-B12 extracted from liver homogenates. (Mean RCN of 6 extractions=1.08, S.D. 0.086, S.E. 0.035). Localization of standard OH-B12 and CN-B12 is underlined. Right: Standard radio-CN-B12 as given to animal. Left: Extracted radiocobalamin is chromatographically identical to OH-B12.

Fig. 3.—Effect of KCN on paper chromatography of radio B12 extracted from liver homogenates after parenteral administration of radioactive CN-B12. Left: Extracted radioactivity from liver. Right: Same after addition of KCN. (Mean R_{CN} of 13 experiments before addition of KCN 0.42, S.D. 0.064, S.E. 0.018, and of 3 experiments after addition of KCN 1.12, S.D. 0.053, S.E. 0.031). Localization of standard OH-B12 and CN-B12 is underlined.

CN-B12 parenterally, 100 per cent of the recovered radioactivity was OH-B12 only 5 days after the final (or 8 days after the first) injection. On the assumption that half the radioactivity was localized to the liver and the latter weighed 27 Gm., the in vivo conversion of CN-B12 in the liver amounted to approximately 0.1 mg. per day per gram (Table 2). In vitro 0.1–0.4 mg. CN-B12 per gram liver per 24 hours was converted to OH-B12 (Table 1).
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DISCUSSION

Method

In this study, only the chromatographic identity of B$_{12}$ forms extracted from tissue was determined. Radioactivity is known to persist as microbiologically active cobalamin.$^{6,8}$ This, together with previous observations of CN-B$_{12}$ conversion, suggests that the radioactivity is identical to that standard cobalamin having the same $R_{cn}$ value.

The low chromatographic mobility in samples where B$_{12}$ had only been liberated with enzymes, but not extracted, was probably due to the interference of the amino-acid-peptide-salt-mixture present in the "liberated" samples, but removed during extraction. This explanation is supported by the fact that even radio-B$_{12}$ added in vitro to the liver homogenate had slower mobility than the same vitamin without the homogenate. Why the digestion of the bound B$_{12}$ with papain prior to incubation-conversion seemed to facilitate conversion is not clear.

The surprising and, in part, statistically significant variation between the different commercial vitamin B$_{12}$ preparations was quite alarming. Differences in additives or in purity were considered.

Some animal-to-animal variations in the conversion rate are to be expected in studies of this kind. It is believed that these variations explain why sometimes a smaller percentage of the CN-B$_{12}$ was converted to OH-B$_{12}$ after 15 days than after 10 days (Table 2).

The present method does not permit recovery of coenzyme forms of B$_{12}$,$^{9}$ but coenzyme B$_{12}$ is apparently recovered as OH-B$_{12}$. It is quite likely, nevertheless, that CN-B$_{12}$ in therapeutic use is converted to a coenzyme form.

Results

When the present studies commenced, no pharmaceutical preparations of OH-B$_{12}$ for therapeutic use were yet available. However, the results explain the therapeutic and metabolic differences since found between OH-B$_{12}$ and CN-B$_{12}$. After the parenteral administration of a therapeutic dose of either CN-B$_{12}$ or OH-B$_{12}$, a much more rapid excretion of CN-B$_{12}$ than of OH-B$_{12}$ is noted initially. The present results with human urine suggest that this is due to the excretion of nontransformed cyanocobalamin. At later times, however, no difference could be found between the excretion rates of the two compounds.$^{12}$ The present results indicate that this is due, in part, to the conversion of the administered CN-B$_{12}$ to the more physiologic OH-B$_{12}$.

It will be interesting to see whether the biologic attributes of the vitamin are wholly or partially dependent on this conversion, whether there are conditions under which transformation cannot occur, and whether conversion takes place in tissues other than liver. Enzymatic aspects of the transformation of CN-B$_{12}$ are also under study.

SUMMARY

Results are presented which indicate that in guinea pigs cyanocobalamin undergoes conversion to hydroxocobalamin, in vivo and in vitro, at the approxi-
mate rate of 0.1–0.4 m\(\mu\)g./day/Gm. of liver. In boiled liver, no conversion was found.

The radioactivity excreted in human urine the first 12 hours after a therapeutic cyanocobalamin dose, on the other, is still cyanocobalamin. It is suggested that demonstrated metabolic differences between therapeutically used cyanocobalamin and hydroxocobalamin are explained, in part, by the time needed to convert cyanocobalamin to hydroxocobalamin.

**SUMMARIO IN INTERLINGUA**

Es presentatae observationes que indica que cyanocobalamina es convertite ad in hydroxocobalamina in le porco de India, in vivo e in vitro, a un rhythmio de approximativemente 0,1–0,4 m\(\mu\)g/die/g de hepate. In hepate bullite, nulle conversion esseva trovate.

Le radioactivitate excernite in urina human durante le prime 12 horas post un dose therapeutic de cyanocobalamina, del altere latere, reflecte ancora le presentia de cyanocobalamina. Es suggestionate que demonstrate differentias metabolic inter cyanocobalamina e hydroxocobalamina in uso therapeutic es explicable, in parte, per le intervallo de tempore requirite per le conversion de cyanocobalamina ad in hydroxocobalamina.

**REFERENCES**

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