REFERENCES

(1) Bardos, T. J., Herr, R. R., and Enkoji, T., *J. Am. Chem. Soc.*, **77**, 960(1955).
(2) Bardos, T. J., Segaloff, A., and Ambrus, J. L., *Nature*, **183**, 612(1959).
(3) Bardos, T. J., Baranski, K., and Czebotar, V. C., unpublished data.
(4) Bardos, T. J., Kotick, M., and Szantay, C., *Tetrahedron Letters*, **1966**, 1759.
(5) Cleland, W. W., *Biochemistry*, **3**, 480(1964).
(6) Klotz, I. M., and Carver, B. R., *Arch. Biochem. Biophys.*, **95**, 540(1961).
(7) Herr, R. R., Enkoji, T., and Bardos, T. J., *J. Am. Chem. Soc.*, **78**, 401(1956).
(8) Gage, J. C., *J. Chem. Soc.*, **1949**, 1148.
(9) Bordwell, F. G., and Andersen, H. M., *J. Am. Chem. Soc.*, **75**, 6019(1953).
(10) Shugar, D., and Fox, J. J., *Biochim. Biophys. Acta*, **9**, 199(1952).
(11) Price, C. C., and Oae, S., "Sulfur Bonding," The Ronald Press Co., New York, N. Y., 1962, pp. 9, 15.
(12) Albert, A., and Phillips, J. N., *J. Chem. Soc.*, **1956**, 1294.
(13) Fox, J. J., and Shugar, D., *Biochim. Biophys. Acta*, **9**, 369(1952).

Absorption, Distribution, and Elimination of a Long-Acting Vitamin B_{12} Preparation

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The prolonged effect of various long-acting vitamin B_{12} preparations has been examined on rabbits regarding absorption, distribution, and elimination after intramuscular injection.

RECENT investigations (13) have shown that patients with pernicious anemia have considerably greater requirements for vitamin B_{12} than have previously been assumed. More recently, there has been considerable interest in preparations that can meet the requirements more adequately and in a more satisfactory way than the aqueous solutions of vitamin B_{12} used previously. The authors have investigated the characteristics of various preparations made on the basis of various principles. A preparation containing cyanocobalamin–tannin complex suspended in aluminum monostearate oil gel[1] was studied in detail.

The clinical value of this preparation is reflected in papers by Bastrup-Madsen *et al.* (4, 5), Schwartz *et al.* (19, 20), Meulengracht (14, 15), an editorial (8), Nielsen and Vedsø (16), Schrumpf (18), and Gough *et al.* (10).

EXPERIMENTAL

Materials and Methods

Preparations A–G were investigated (Table I). After intramuscular injection (i.m.) in rabbits, the following were investigated: (*a*) liberation of vitamin $B_{12}{}^{60}Co$ from the site of injection; (*b*) absorption of vitamin $B_{12}{}^{60}Co$ by the liver; and (*c*) excretion of vitamin $B_{12}{}^{60}Co$ in urine and feces.

For preparations F and G the investigations were supplemented by radioactivity counts and microbiological determinations of vitamin B_{12} in liver, kidney, and femoral muscle about 3 months after the start of trial.

Preparations

A number of preparations were made using vitamin B_{12} labeled with ^{60}Co. Their composition is shown in Table I. The products were prepared according to methods described in a British patent (6).

The distribution and excretion of vitamin B_{12} after parenteral administration of preparation B and of aqueous solutions of cyanocobalamin were investigated in rats and in healthy subjects by Davis *et al.* (7), Thompson and Hecht (22), Astudillo *et al.* (3), and Glass *et al.* (9), but no records have been found of investigations on patients with pernicious anemia. The authors have not investigated any preparations of vitamin B_{12} suspended in oil or vitamin B_{12} suspended in 2% monostearate oil gel; the latter was described by Arnold *et al.* (1, 2), Sobell *et al.* (21), and Heinrich and Gabbe (11). It does not appear to possess any retarded action of interest for clinical use.

Preparations F and G correspond to a marketed suspension of cyanocobalamin–tannin complex in aluminum monostearate oil gel,[1] except that labeled vitamin B_{12} (^{60}Co) was used instead of ordinary cyanocobalamin. Spectrophotometric and microbiological checks were made on the preparations. The radiochemical purity of the labeled compounds was confirmed by paper chromatography and subsequent counts and by microbiological determinations of the vitamin B_{12} activity on agar plates with *Lactobacillus leichmannii*, as described by Winsten and Eigen (23), among others.

Animal Material.—Twelve white male rabbits were used. The initial weights were from 2.7 to 3.0 Kg. The rabbits were anesthetized with sodium amobarbital (30 mg./Kg.) supplemented by ether during the radioactivity counts.

Standards and Methods of Measurement.—The radioactivities above the site of injection and above the liver were determined on a scintillation detector shielded by 5 cm. of lead with a 30-mm. round opening upon which the object to be measured was placed. Feces and organs were homogenized before

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[1] Marketed as Betolvex by Dumex Ltd., Copenhagen, Denmark.

TABLE I.—DOSAGE SCHEDULE OF VARIOUS VITAMIN B_{12} PREPARATIONS

Prepn.	Form of Vitamin B_{12}	Vehicle	Vitamin B_{12} Concn., mcg./ml.	B_{12}-^{60}Co Concn., μc./ml.	Quantity Injected, ml.
A	Cyanocobalamin	Aqueous 0.9% NaCl soln.	500	0.18	0.30
B[a]	B_{12}–zinc–tannate	Aqueous suspension	500	0.18	0.30
C	B_{12}–zinc–tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
D[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
E	B_{12}–tannate	Suspended in sesame oil	500	0.5	0.25
F[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50
G[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50

[a] Trademarked preparation is Depinar. [b] Trademarked preparation is Betolvex.

counting. A scintillation detector with a well-type crystal was used for counting radioactivity in urine and liquid samples of feces and organs.

The microbiological vitamin B_{12} activity was determined by the *L. leichmannii* method (12) as modified by Noer (17).

Before and after each measurement the scintillation detector was checked against a standard and corrected accordingly.

In order to determine the absolute content of radioactive vitamin B_{12} in samples of urine, feces, and organs, a known quantity of the injection preparation in question was added after the samples had been counted. Thus, the counts of the samples and of the standards were carried out under the same conditions.

Trial Methods.—Blank values were determined on urine and feces collected for 24 hr. from the rabbits in specially constructed metabolism cages. After being anesthetized, the rabbits were strapped to the counting table with the right femoral muscle above the detector.

After the background had been counted, the preparation to be tested was injected intramuscularly into the right femoral muscle directly above the detector. The doses employed are shown in Table I. Immediately afterward, the radioactivity at the site of injection was screened at various sites around the injection site, and the counts were carried out at the site of highest activity. Immediately afterward, the radioactivity in the hepatic region was determined in the manner described above.

Counts of the activity at the site of injection and in the hepatic region were carried out at irregular intervals for 3 months, and their relative values, as percentages of the initial count, were calculated.

During the first 3–4 days after the injection, urine and feces were collected as 24-hr. samples.

In the tests made with preparations F and G, the rabbits were killed with ether after the test period of about 3 months; liver, kidney, and femoral muscle were dissected free, and their contents of radioactive material were determined. In addition to the organs mentioned above, the spleen, heart, bladder (containing urine), small intestine (duodenum, jejunum, ileum), large intestine (cecum, including vermiform appendix, colon, rectum), and contents of both large and small intestine were collected from rabbit *3*. (See Table II.) Liver, kidneys, and muscle from the right femur were taken from the control rabbit, which had been kept on the same diet as the test animals for 3 months, in order to obtain a basis of comparison and a standard for the content of vitamin B_{12}. A microbiological determination of vitamin B_{12} was carried out on all homogenates of liver, kidney, and femoral muscle.

RESULTS

Measurement Above the Site of Injection.—Figure 1 shows the increase of prolonged effect obtained by combining the different principles. It will be seen that a clearly prolonged effect is only obtained by suspending a vitamin B_{12} compound in

TABLE II.—PERCENTAGE RADIOACTIVITY ABOVE SITE OF INJECTION

Rabbit	Prepn.	Std.	Days After Injection 2	3	4	7	9	10	11	15	18	21	25	28	30
1	F	100	69.8		65.7				60				47.9		
2	F	100		76.2			68.2					52.7			
3	G	100	86.5					85		81.5				72	
4	G	100		61.1		55.1			50		40.5				34.6

TABLE II.—(*Continued*)

Rabbit	Prepn.	Std.	Days After Injection 37	39	44	46	53	59	66	78	87	95
1	F	100		34			22.7			9.7		
2	F	100	38.9						19.9		7.6	
3	G	100			54.6							
4	G	100				25.5		19.4				4.1

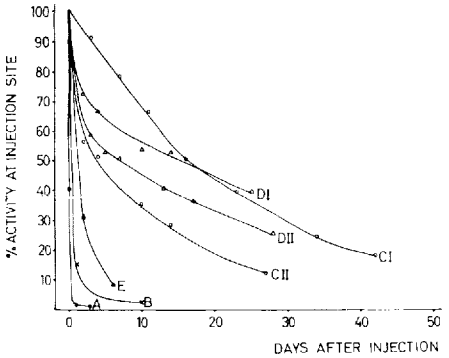

Fig. 1.—Absorption of radioactive vitamin B_{12} from the site of injection. Key: A, aqueous solution of vitamin B_{12}; B, aqueous suspension of B_{12}–zinc–tannate; C_I and C_{II}, B_{12}–zinc–tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel; E, B_{12}–tannate suspended in oil.

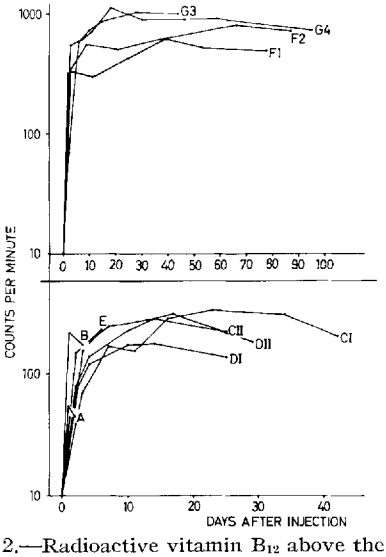

Fig. 2.—Radioactive vitamin B_{12} above the liver. Key: (bottom) A, aqueous vitamin B_{12} solution; B, aqueous suspension of B_{12}–zinc–tannate; C_I and C_{II}, B_{12}–zinc–tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel. E, B_{12}–tannate suspended in oil. Key: (top) F_1 and F_2, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel; G_3 and G_4, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel.

aluminum monostearate oil gel. Table II shows the prolonged effect of two preparations of vitamin B_{12}–tannate in aluminum monostearate oil gel (F and G). Rabbit *3*, which died during narcosis about 1.5 months after the beginning of the investigation, differed from the other three. During the period of investigation the rabbit did not move about much. This could have been the reason for the small liberation of vitamin B_{12} from its intramuscular depot.

Measurement Above the Liver.—Figure 2 gives an impression of the storage of vitamin B_{12} in the liver, obtained by combining the different principles. The graphs for preparations F and G show considerably higher counts because of the larger amount of radioactivity injected (see Table I).

The counts shown in Fig. 2 were obtained by deducting the value expressing a scattered radiation from the depot of the femoral muscle from the counts corrected for background. The value of scattered radiation decreases in proportion to the activity at the site of injection; accordingly, the correction will be:

(corr. counts per minute above liver immediately after injection) × (% activity at site of injection on the day in question) divided by 100.

Because of the somewhat inaccurate method of measurement, only the shapes of the graphs, not the absolute values should be compared.

Excretion in Urine.—Table III shows the difference in excretion in urine after administering the various preparations. The difference in urinary excretion between preparation D and preparations F and G may be explained partly by the larger quantity of F and G injected, and partly by a minute amount of free vitamin B_{12} in the preparations.

Excretion in Feces.—The excretion in the feces was <1%/24 hr.

Content of Vitamin B_{12} in Liver, Kidneys, and Femoral Muscle.—At the time when the rabbits were killed the vitamin B_{12} contents of liver, kidneys, and femoral muscle were measured, both by radioactivity measurements and by microbiological determinations. Table IV shows the results. The counts immediately show that the content of vitamin B_{12} in liver and kidneys of rabbits treated with preparations F and G has increased compared with that of the control rabbit. The content of vitamin B_{12} in the right femur is due to vitamin B_{12} not yet liberated from the site of injection, and a reasonable agreement with the results obtained by radioactivity measurements above the site of injection was found. The difference between vitamin B_{12} measured microbiologically and vitamin B_{12} determined by radioactivity gives us the level of vitamin B_{12} in the rabbits before the injection and is of the same order of magnitude as those obtained microbiologically from the control rabbit.

Radioactive Vitamin B_{12} Content in Other Organs and Tissues (Rabbit 3).—*Spleen.*—No measurable vitamin $B_{12}{}^{60}Co$ (<1% of dose administered).

Heart.—No measurable vitamin $B_{12}{}^{60}Co$ (< 1% of dose administered).

Contents of Intestine.—Less than 1% of dose administered.

TABLE III.—URINARY EXCRETION OF VARIOUS VITAMIN B_{12} PREPARATIONS

Rabbit	Prepn.	1st. 24 hr., %	2nd. 24 hr., %	3rd. 24 hr., %	4th. 24 hr., %
	A	60	<5	<2	...
	B	10–15	<5	<2	...
	C	<1	<1	<1	...
	D	<1	<1	<1	...
	E	19	11	2	...
1	F	7	6	<1	<1
2	F	13	1	<1	<1
3	G	2	4	<1	<1
4	G	6	1	<1	<1

TABLE IV.—VITAMIN B_{12} CONTENTS OF LIVER, KIDNEYS, AND RIGHT FEMORAL MUSCLE

Prepn.	F		G		
Rabbit	1	2	3	4	5 (Control)
Days after inj.	78	87	44	95	...
Liver					
Weight, Gm.	184	151	71	168	124
Vitamin B_{12}, microbiologically, total, mcg.	66.8	65.3	35.2	81.5	30.1
Total vitamin B_{12} radioactivity, mcg.	28.9	23.6	23.9	49.1	...
Kidneys					
Weight, Gm.	13.7	13.9	13.5	14.8	19.3
Vitamin B_{12}, microbiologically, total, mcg.	6.2	7.5	6.0	7.4	4.2
Total vitamin B_{12} radioactivity, mcg.	3.2	3.0	2.5	4.4	...
Right Femoral Muscle					
Weight, Gm.	101	126	98	131	112
Vitamin B_{12}, microbiologically, total, mcg.	67	26	...	30	8
Total vitamin B_{12} radioactivity, mcg.	60	23	275	24	...
Radioactivity above site of injection, % (see Table II)	12% of dose 9.7	4.6% of dose 7.6	55% of dose 54.6	4.8% of dose 4.1	...

Small Intestine (*Jejunum, Duodenum, Ileum*).—Less than 1% of dose administered.

Large Intestine (*Cecum, Including Vermiform Appendix, Colon, Rectum*).—Less than 1% of dose administered.

Bladder (*Containing Urine*).—Less than 1% of dose administered.

DISCUSSION

These investigations on rabbits clearly reveal that preparations of B_{12}–tannate or B_{12}–zinc–tannate in a 2% aluminum monostearate oil suspension release vitamin B_{12} from an intramuscular depot over a much longer period of time than does B_{12}–zinc–tannate in an aqueous suspension or B_{12}–tannate in an oil suspension or vitamin B_{12} in a water solution (Fig. 1).

Vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension can release vitamin B_{12} for the body for more than 1 month compared with a few days for the other types of preparations investigated.

By this prolonged release one avoids the urinary excretion that always follows injections of vitamin B_{12} in excess of serum vitamin B_{12} binding capacities. This also means that vitamin B_{12} is available for storage in vitamin B_{12} depots over a longer period of time (Fig. 2). Due to the techniques used, only the shapes of the curves are comparable. A more detailed study of 2 different vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension showed vitamin B_{12} was released from the intramuscular depots for more than 2 months, with some of the injected material still left at the injection site as vitamin B_{12} as determined by microbiological methods. Table IV shows there is good agreement between direct measurements above the site of injection and measurements of the dissected femoral muscle.

Furthermore, it is shown (Table IV) that the released vitamin B_{12} was deposited in the liver and kidneys, as could be expected, and that the liver and kidneys from rabbits injected with B_{12}–tannate in a 2% aluminum monostearate oil suspension contained a considerably greater amount of vitamin B_{12} in comparison with the control.

SUMMARY

By investigations carried out on rabbits, the properties of various vitamin B_{12} preparations with prolonged effect are demonstrated, including the liberation from intramuscular depots and the storage in the natural depots of the body. After intramuscular injection of B_{12}–tannate in aluminum monostearate oil gel the observations were: (*a*) a prolonged liberation of vitamin B_{12} from the depot injected into the muscle; (*b*) an increased vitamin B_{12} activity in liver and kidneys, taken as a sign of storage in the natural depots of the body; and (*c*) minimum excretion in urine.

REFERENCES

(1) Arnold, A., *et al.*, *Am. J. Clin. Nutr.*, **10**, 56(1962).
(2) Arnold, A., *et al.*, *Am. Practitioner Dig. Treat.*, **9**, 1835(1958).
(3) Astudillo, M., *et al.*, *Rev. Clin. Espan.*, **78**, 68(1960).
(4) Bastrup-Madsen, P., *et al.*, *Ugeskrift Laeger*, **124**, 1687(1962).
(5) *Ibid.*, **128**, 261(1966).
(6) Brit. pat. 960030.
(7) Davis, R. L., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **101** 211(1959).
(8) Editorial, *Ugeskrift Laeger*, **124**, 1710 (1962)
(9) Glass, G. B. J., *et al.*, *Blood*, **18**, 511(1961).
(10) Gough, J., *et al.*, *Lancet*, **ii**, 1311(1964).
(11) Heinrich, H. C., and Gabbe, E. E., *Arzneimittel-Forsch.*, **11**, 1087(1961).
(12) Hoff-Jørgensen, E., "Microbiological Assay of Vitamin B_{12}," in Glick, D., ed., "Methods of Biochemical Analysis," vol. 1, Interscience Publishers, Inc., New York, N. Y., 1957, pp. 81–113.
(13) Kristensen, K., *et al.*, *Ugeskrift Laeger*, **124**, 1681 (1962).
(14) Meulengracht, E., *ibid.*, **124**, 1693(1962).
(15) Meulengracht, E., *Lancet*, **i**, 354(1963).
(16) Nielsen, E. G., and Vedsø, S., *Ugeskrift Laeger*, **125**, 1446(1963).
(17) Noer, B., "Vaekstfaktorer for *Lactobacillus leichmannii* 313 i Halv-og Helsyntetiske Substrater," thesis in "Collected Papers from Ferrosan," vol. 3, I.D.O., Copenhagen, Denmark, 1956–1959, 120 pp.
(18) Schrumpf, A., *Tidsskr. Norske Laegeforen.*, **83**, 1541 (1963).
(19) Schwartz, M., *et al.*, *VII Intern. Kongr. Inn. Med.*, **1962**, 561.
(20) Schwartz, M., *et al.*, *Lancet*, **ii**, 1181(1962).
(21) Sobell, S. D., *et al.*, *Am. J. Clin. Nutr.*, **8**, 214(1960).
(22) Thompson, R. E., and Hecht, R. A., *ibid.*, **7**, 311(1959).
(23) Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, **181**, 109(1949).