Summary. A comparison of α and β lipoprotein cholesterol levels of lipoproteins of human serum obtained by dextran sulfate precipitation compares well with analyses of lipoproteins obtained by ultracentrifugation. Comparison of dextran sulfate from 2 different sources gave identical results. The presence of oxalate may interfere with the analysis of β lipoprotein cholesterol, but this can be avoided by using analytical methods which entail digitonide precipitation.

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Tissue Distribution and Storage Forms of Vitamin B_{12} Injected and Orally Administered to the Dog.* (28011)

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Previous attempts by the present authors (1,2,3) to compare the behavior of orally administered Vit. B_{12} with that of injected material have been inconclusive. Thus, 30 days after administration to normal humans, 0.19% of a tracer amount (0.5 μg) of injected radioactive Vit. B_{12} is excreted(1) per day; and in an independent study(2), the eventual daily output was found to be 0.23% of a 3 μg dose. By contrast the total excretion rate of normal Vit. B_{12} from body stores (3) appears to be only $\simeq$ 0.03% per day. Such a divergence may result from incomplete mixing of radioactive Vit. B_{12} and body stores of the vitamin. Alternatively it may actually reflect the functioning of different compartments attending the several modes of administration involved. The possibility of degradation or transformation of vitamin within tissues and organs must also be considered. Because of the apparent discrepancy, experiments were initiated in humans

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and in dogs in which Vit. B_{12} was administered orally and by injection to the same test subject. A double labeling procedure was employed to permit simultaneous observations of both modes of administration, thus ruling out subject variability. By this means it should be possible to ascertain the role of mode of administration. This paper reports measurements of comparative retention by various tissues of the dog and the nature of the retention form of the vitamin in the liver.

Materials and methods. Vit. B_{12} preparations labeled with cobalt-57 and with cobalt-60 were administered to 2 dogs (mongrels; weights $\simeq$ 10 kg) for a period of about 2 weeks. During this period each animal received 22 oral doses of Vit. B_{12}-Co^{57}, each containing 0.11 μg (and 0.6 μc) of vitamin administered in frozen gelatin capsules, and 7 intravenous injections of $\simeq$ 2.1 μg ($\simeq$ 2.0 μc) of Vit. B_{12}-Co^{60}. Total Vit. B_{12} fed was 2.4 μg (13.2 μc) orally and 14 μg (13 μc) intravenously.

The dogs were sacrificed 4 weeks after receiving the last dose. Weighed samples from all organs and tissue were digested in 2 ml of 6N NaOH, and measured in a well type scintillation detector by means of a single channel gamma ray spectrometer.

Storage forms of Vit. B_{12} were studied by the reverse isotope dilution method(4,5). Portions (10-15 g) of liver were homogenized with water, and the radioactive components liberated either by acid nitrite or by enzymatic digestion, in the presence of crystalline cyanocobalamin (Vit. B_{12}) and/or hydroxocobalamin added previously as carriers ($\simeq$ 1 mg of each cobalamin). Cyanocobalamin and/or hydroxocobalamin fractions were then isolated, and purified for measurement of recovered vitamin (spectrophotometrically) and of associated Co^{57} and Co^{60} (gamma ray scintillation spectrometry). The percentage of initial radioactivity recoverable as the 2 cobalamins may be computed from the fractional recovery of radioactivity and vitamin in the purified fractions. Details of isolation and purification procedures, as well as calculations involved in such dilution procedures, have been published elsewhere(4,5,6,7). Papain(8) and trypsin(9) were employed for enzymatic digestion.

Results. Tissue radioactivities in cpm per gram due to Co^{57} and Co^{60} observed in 47 different tissue samples from each of 2 dogs are reported graphically in Fig. 1, which shows log-log plots of the tissue specific Co^{60} activities and the corresponding Co^{60} values.‡ The slopes of these plots indicate that, with relatively few exceptions the average ratio of Co^{57}/Co^{60} is approximately constant in all tissue specimens examined. This ratio has an average value of 8 for both animals. Taking into account the value of 19.3 for the ratio of specific radioactivities, in cpm per μg of Vit. B_{12}-Co^{57} to that of Vit. B_{12}-Co^{60} under respective conditions of spectral measurement, one computes that the average relative amounts of injected Vit. B_{12} (-Co^{60}) retained by tissue are 2.4 times the corresponding values for orally administered Vit. B_{12} (-Co^{57}). This is in accord with normal expectation for these modes of administration.

Results of reverse isotope dilution assays of both dog livers are recorded in Table I. To insure equilibration of labeled vitamin in tissue with carrier vitamin, samples were boiled for 15 minutes or 1-2 hours after enzymatic digestion prior to isolation of cobalamins instead of the customary 5 minutes (for trypsin) or 15 minutes (for papain) to destroy excess enzyme. Treatment of both specimens with nitrous acid in the presence of cyano carrier indicates that all of the radioactivity can be isolated as the cyanocobalamin. This is in agreement with a previous report(4). However, use of carrier hydroxocobalamin indicated that most of the radioactivity could also be recovered as the hydroxocobalamin. Obviously, use of nitrite in treating tissue samples leads to an equilibration of radioactivity with either of the carrier cobalamins. Accordingly, nitrite will not maintain the integrity of the several cobalamins in tissue, but serves only to indicate a total cobalamin radioactivity. A similar effect was noted in a blank experiment with sodium metabisulfite(10) added to a

‡ A preliminary report of this observation was made in Ref. 3.

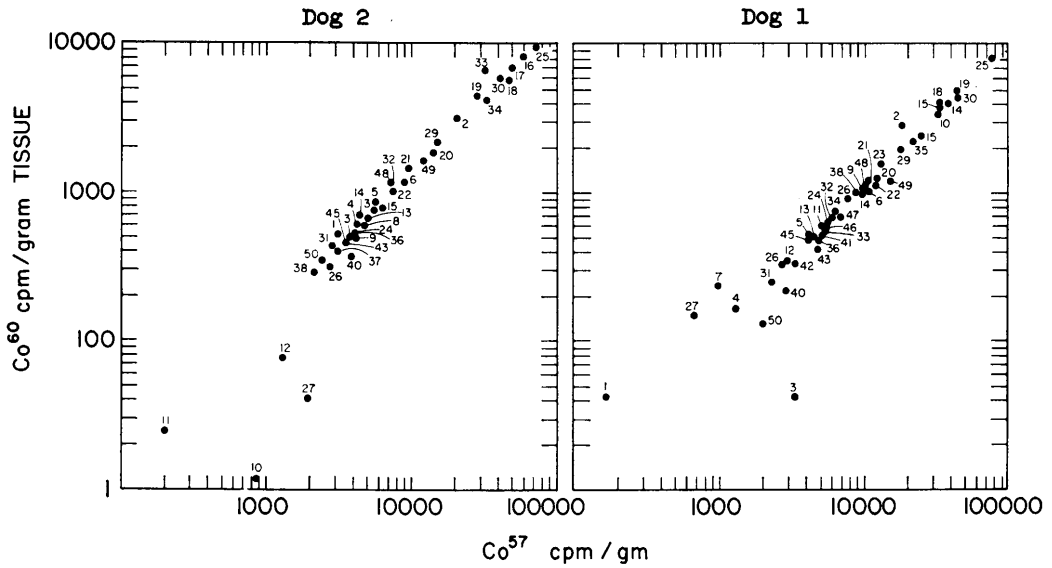

FIG. 1. Radioactivities in tissues of 2 experimental animals receiving oral vitamin B_{12}-Co^{57} and intravenous vitamin B_{12}-Co^{60}. Numbers indicate the following tissues:

1. Aorta
2. Heart
3. Skin
4. Skeletal muscle
5. Diaphragm muscle
6. Thyroid
7. Parathyroid
8. Lung
9. Thymus
10. Cartilage (sternum)
11. Bone
12. " marrow
13. Urinary bladder
14. Gall "
15. Spleen
16. Kidney
17. Adrenals
18. Pancreas
19. Liver
20. Submaxillary gland
21. Axillary lymph node
22. Mesenteric lymph node
23. Cerebrum
24. Thoracic duct
25. Pituitary
26. Femoral nerve
27. Cornea
29. Testis
30. Prostate
31. Esophagus
32. Stomach-cardia-wall
33. Stomach-fundus-mucosa
34. Stomach-fundus-wall
35. Stomach-pylorus-wall
36. Duodenum-mucosa
37. Duodenum-wall
38. Jejunum-mucosa
40. Ileum-mucosa
41. Ileum-wall
42. Cecum-mucosa
43. Cecum-wall
45. Colon-wall
46. Rectum-wall
47. Tonsil
48. Popliteal lymph node
49. Parotid
50. Omentum fat

mixture of inactive livers, papain, radioactive hydroxocobalamin-Co^{57} and cyanocobalamin-Co^{60} and both cobalamins added as carriers.

Differentiation between the cobalamins is possible, however, by digesting tissue with papain to liberate radioactive components. Papain treatment of the liver of Dog 1 in the presence of both carriers yields a cyanocobalamn fraction accounting for 7-21% of total tissue radioactivity§ and a hydroxocobalamin fraction containing 66-100% of tissue radioactivity. Furthermore the percentages of administered cobalt-57 and cobalt-60 recovered in the cobalamin fractions appeared to be the same. Subsequent examination of these fractions by chromatography on paper indicated high purity; and reprocessing of eluates from paper by solvent extraction and ion exchange resins yielded identical recovery results. Similar enzymatic treatment of liver from Dog 2 in presence of cyanocobalamin carrier yielded a cyanocobalamin fraction with 10-15% of total tissue radioactivity, and with hydroxocobalamin carrier, a hydroxo-fraction with $\simeq$ 100% of initial activity. In the presence of both carriers, values were 7-26% in the cyanocobalamin fraction, and 62-100% in the hydroxocobalamin isolate. In all instances, cobalt-57 and cobalt-60 were comparable in behavior.

A limited number of trypsin digestions also indicated transfer of cyanocobalamin radioactivity to hydroxocobalamin. However, cobalamin extraction after trypsin treatment

§ A preliminary report of this observation was made in 2nd European Symposium on Vitamin B_{12} and Intrinsic Factor, Hamburg, Aug. 2-5, 1961. Enke Verlag, Stuttgart, 1962, p306.

TABLE I. Reverse Isotope Dilution Assay of Dog Livers.

Dog	Tissue treatment	Carriers	Exp series	Boiled (min)	% recovered as Cyanocobalamin Co^{57}	Cyanocobalamin Co^{60}	Hydroxocobalamin Co^{57}	Hydroxocobalamin Co^{60}
1	HNO_2	Cyano	A	60	99.6	97.3	—	—
		Hydroxo‡	M	60	—	—	95.0	95.0
	Papain	Cyano	H*	15	6.7	7.4	101	108
		Hydroxo	H*	60	19.7	21.4	66.6	66.4
2	HNO_2	Cyano	A	60	97.6	97.8	—	—
		Hydroxo‡	J	60	—	—	92.7	92.7
	Papain	Cyano	C	15	9.6	10.8	—	—
		"	C	15	16.5	13.8	—	—
		Cyano + hydroxo	D	15	6.6	6.2	100	100
			F*	60	25.2	26.1	64.5	60.6
			E	60	17.7	17.1	70.4	69.2
			E	120	22.8	21.7	62.1	61.5
		Hydroxo‡	J	60	—	—	100	105
	Trypsin	Cyano	B	15	45.2	47.2	—	—
		Cyano + hydroxo	G†	60	39.0 (20.0)	38.2 (26.9)	45.0	46.3
		Hydroxo	L	60	—	—	118	112

* These isolates were purified further by chromatography on paper, elution, solvent extraction, and ion exchange resins. Results obtained were essentially the same as in the standard procedure.

† Further purification of the cyanocobalamin fraction yielded the lower results shown in parentheses.

‡ Plus trace of cyanocobalamin $\simeq$ 1% of hydroxocobalamin carrier weight.

was much more difficult, and required prolonged boiling, indicating incomplete liberation of cobalamins (from binding proteins?). This is borne out by the lower results obtained in Exp. G (Table I) after further purification of isolates by chromatography on paper. This equivocal behavior was not observed with papain.

Discussion. In an earlier investigation(4) of the stability of injected Vit. B_{12}, it was concluded that "Vitamin B_{12}-Co^{60} is retained by dog livers as an intact cobalamin, probably as cyanocobalamin." It now appears that this result reflects the method of treating tissue rather than the stability of cyanocobalamin, since the use of nitrite, and probably other reducing agents, does not assure the inviolability of the cobalamin structures. Nitrous acid may be used in the isotope dilution assay for total cobalamins; it will not serve to differentiate between cyano- and noncyanocobalamins in tissue.

Differentiation was possible by subjecting tissue to enzymatic digestion. By this means it was demonstrated that the bulk of the administered cyanocobalamin is converted during storage in liver to a noncyano form of cobalamin which is isolable as hydroxocobalamin. Whether this conversion is limited to the hydroxo-stage, or whether conversion proceeds to the cobamide coenzyme(11,12), is not discernible from these experiments since isolations were not performed in the dark; and the cobamide coenzyme could easily have degraded to hydroxocobalamin(12). That conversion of cyanocobalamin to its coenzyme does occur in the rabbit was reported by Fenrych *et al.*(13).

The similarity in behavior of oral and injected Vit. B_{12} is worthy of note. Cobalt-57 and cobalt-60 are similarly distributed between the 2 cobalamin fractions isolated from the liver, indicating identical conversion of oral and parenteral Vit. B_{12} retained in this organ. This is in accord with the essential constancy of the Co^{57}/Co^{60} in all tissues. This suggests that Vit. B_{12} and conversion products retained by the animal are incorporated in a common pool regardless of mode of administration. Cooperman *et al.*(14) have already reported that oral Vit. B_{12}-Co^{60} injected subcutaneously into dogs equili-

brates in similar fashion with endogenous tissue vitamin by 14 days after administration. Comparable results were obtained by Gräsbeck *et al.*(15) with rats. One must conclude that the different fractional excretion rates of injected and endogenous Vit. B_{12} are not determined by differences in mode of administration. A possible explanation lies in the rate of conversion of administered cyanocobalamin to noncyanocobalamins, cobamide coenzyme and/or bound forms of either. Thus Doscherholmen and Hagen(16) have reported 5 components in urinary excretion by the human. Chemical changes such as those discussed, and not necessarily different physical compartments, could account in part for the complex nature of the excretion pattern observed with Vit. B_{12}.

Summary. 1. The distribution of oral and intravenous Vit. B_{12} was determined simultaneously in dogs using vitamin preparations labeled respectively with cobalt-57 and cobalt-60. 2. Thirty-one days after administration, the ratio of Co^{57}/Co^{60} was constant in all tissues, indicating the existence of a common pool for cobalamin and related compounds regardless of mode of administration. 3. Conversion of administered labeled Vit. B_{12} to a noncyano form was demonstrated by reverse isotope dilution analysis of livers. This is true regardless of mode of administration. 4. It is suggested that chemical changes in the vitamin account in part for the complex excretion pattern of labeled Vit. B_{12}.

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