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Vitamin B_{12} Biosynthesis after Oral and Intravenous Administration of Inorganic Co^{60} to Sheep.* (19586)

R. A. MONROE, H. E. SAUBERLICH,† C. L. COMAR, AND S. L. HOOD.

From the University of Tennessee-Atomic Energy Commission Agricultural Research Program, Oak Ridge

The literature through 1950 concerning the chemistry, physiological role, and clinical use of vit. B_{12} has been reviewed comprehensively by Smith(1), Ungley(2), and Marston(3). The essentiality of cobalt in ruminant nutrition has been recognized for many years(4,5) and a number of distribution studies using inorganic radioactive cobalt have been reported(6-12). Also, some information is available regarding the distribution of vit. B_{12} labeled with radioactive cobalt(12-14). Nevertheless, the relationship between the dietary need of inorganic cobalt and the physiological role of vit. B_{12}, especially concerning the factors involved in B_{12} biosynthesis, has remained little understood.

The present work extends the information obtained in earlier radiocobalt studies by partition of the labeled cobalt into 3 distinct fractions of the excretions and tissues—namely, vit. B_{12}-like, inorganic, and bound. These data have yielded evidence concerning the degree and possible sites of vit. B_{12} biosynthesis in sheep.

Methods. Treatment of animals. Four normal wether lambs weighing about 80 lb each were placed in metabolism stalls. Each of the animals received 2 mc of Co^{60} chloride solution containing about 1.6 mg of cobalt. Two were dosed orally by gelatin capsule and 2 received an intravenous (jugular) injection. The metabolism stalls and the technics for handling the animals, administering the activity, and collecting samples have been described by Hansard, *et al.*(15). *Collection of samples.* Feces and urine were collected continuously and samples were taken daily over a period of a week. The urine was collected under toluene and both feces and urine samples were refrigerated with toluene added. At the end of 7 days the animals were sacrificed and various tissues were sampled for analysis. Aliquots of each sample were counted for total radioactivity. In addition, butanol-soluble and water-soluble phases of similar aliquots were separated and counted as described in detail below. In all samples except urine some residual activity was also obtained in the insoluble fraction.

Counting technics. Several counting technics were employed in the work, depending on the sample size and the amount of radioactivity present in the sample. With large amounts of activity, accurate counts were obtained by measuring a few grams of fresh sample using an unshielded Geiger-Müller tube with an 89.5 mg/cm^2 aluminum absorber, thus recording only gamma activity. With somewhat less activity, it was necessary to count without an absorber. In this case, a wet ash digest was prepared, to assure reproducible geometry, and measured with the unshielded thin-window tube. Still smaller amounts of activity were counted by using dry ashed samples and a shielded thin-window G-M tube.

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† Oak Ridge Institute of Nuclear Studies Fellow on leave from Alabama Polytechnic Institute.

When only minute amounts of activity were present, dry ashed samples were counted in a windowless proportional counter. Data from all methods were reduced to a comparable basis by counting standards from the original dosing solution under each set of conditions and calculating all values in terms of percent of the dose.

Preparation of samples for total radioactivity determinations. The total Co^{60} present in feces was usually determined by counting gamma radiation in 5 to 10 g of the fresh material. In a few samples, however, the count was low enough that solution counting was necessary. For this purpose about 5 g of feces were digested with concentrated nitric acid, the resulting solution made up to 10 ml and transferred to a 50 mm Petri dish for counting. In all urine samples, total radioactivity determinations were made by direct solution counting. For the determination of total activity in tissues, 1-3 g samples were placed in flat, 40 mm, capsule type porcelain crucibles, dried in an oven at 110°C and then ashed at 550°C in a muffle furnace for several hours. The ash was dissolved in a few drops of concentrated HNO_3 and enough water was added to form a thin layer over the bottom of the crucible. The samples were then evaporated to dryness under infra-red heat lamps and counted with a shielded thin-window G-M tube. Self-absorption corrections were made when the mass of the sample to be measured was large enough to produce more than a 5% loss of counts.

Chemical fractionation. The methods used in obtaining Co^{60} fractions from samples were developed from those discussed by Woodruff and Foster(16). For fractionating feces, 5 g samples were homogenized in a Waring blendor with 40 ml of 0.5% sodium phosphate buffer (pH 8.0), 1 ml of toluene, and 20 mg of crystalline trypsin. For tissues, 1 g samples were homogenized in a Potter-Elvehjem homogenizer with 10 mg of crystalline trypsin, a few drops of toluene, and phosphate buffer added to total about 8 ml. After incubation at 37°C for 45 to 120 hours, the homogenates were filtered or centrifuged. The residues were washed thoroughly and the washings added to the supernatant solution. The supernatant was saturated with ammonium sulfate and then extracted four times with equal volumes of *n*-butanol. It was found that under these conditions at least 95% of the butanol soluble Co^{60} was extracted into the butanol phase, while all the water soluble Co^{60} remained in the aqueous phase. The two phases were separated by centrifugation and subsequent removal of the butanol layer with a vacuum siphon. The Co^{60} in the butanol phase will be referred to throughout this paper as "vitamin B_{12}-like." When vit. B_{12} is mentioned without modification, it should be remembered that this is the fraction meant. Actually, paper chromatography of B_{12}-like extracts from several representative samples showed a behavior similar to a solution of pure B_{12} labeled with Co^{60}‡ as contrasted with a solution of inorganic Co^{60}. The Co^{60} in the aqueous phase will be called "inorganic;" that remaining in the residues, "bound." Due to difficulties in manipulation the "bound" values were determined routinely by difference. However, some residues were counted directly and the values obtained agreed closely with those obtained by difference. No residue remained in the urine samples after extraction, which was carried out without preliminary trypsin digestion. Aliquots of all the above butanol and aqueous extracts were dried in 1 inch stainless steel cups, ashed at 450-550°C for an hour or two to reduce the mass, and counted in a windowless proportional counter.

Results and conclusions. In order to avoid confusion, it should be made clear at the outset that the data may be expressed by two distinct quantities: a) a percentage of the dose (*e.g.*, 84.4% of the ingested Co^{60} was excreted in the feces), and b) the percentage of Co^{60} in any given fraction as a function of the total Co^{60} present (*e.g.*, 12% of the Co^{60} found in the feces was vitamin B_{12}-like). The former quantity is used primarily in discussing total amounts of Co^{60} excreted or the concentration in tissues when expressed as percentage of dose per gram of fresh sample; the latter is used in expressing the relationships

‡ The Co^{60}-labeled vit. B_{12} was supplied by Merck & Co., Rahway, N. J.

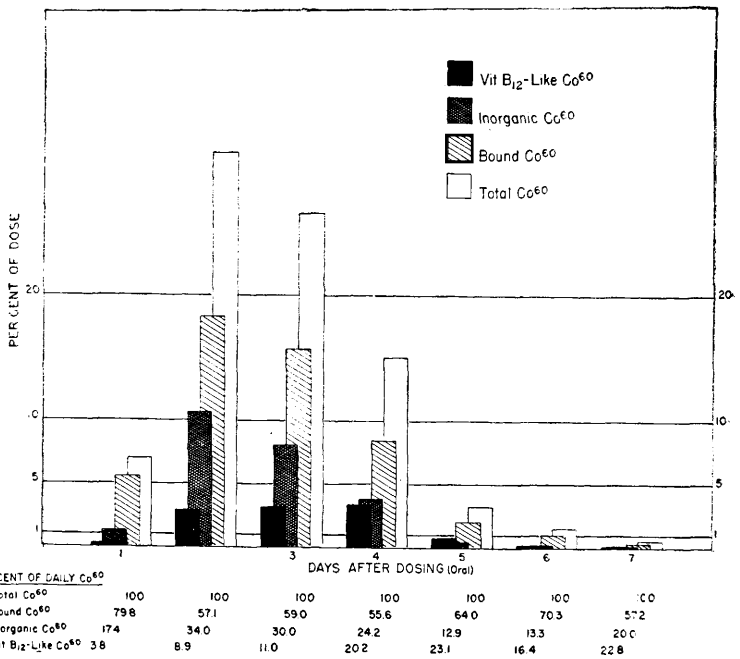
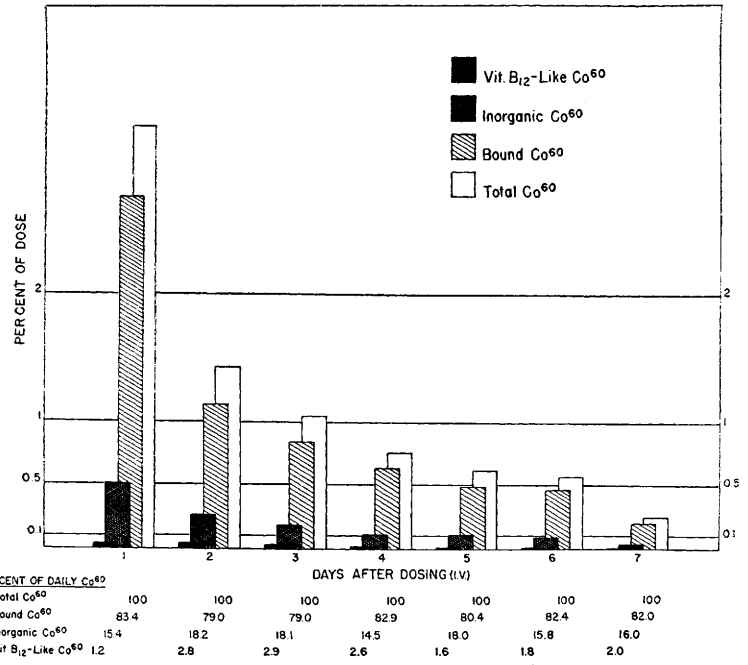


FIG. 1. Average excretion of Co^{60} in the feces of two sheep after oral administration plotted as % of dose/day; the tabulated values indicate the % of total Co^{60} represented by each fraction. The total fecal excretion for the week amounted to 84.4% of the dose.

of the various fractions to one another and to the total amount of Co^{60} present. Average values for the 2 animals are presented in each case since they were in general agreement.

Total excretions. From Fig. 1 and 2 (feces data) and Table I (urine data) it may be noted that 84.4% of the oral cobalt dose was excreted in the feces and 10.8% in the urine. After intravenous injection 8.0% of the dose appeared in the feces and 77.7% in the urine. This small retention and dependence of excretory route upon the mode of administration is in general agreement with values reported for other species(6-11).

Fecal excretion. The details of Co^{60} excretion in the feces after oral administration are presented in Fig. 1 and after intravenous injection in Fig. 2. The graphs denote the percentage of administered Co^{60} excreted daily in each of the chemically characterized fecal fractions. For convenience, values for Co^{60} in each fecal fraction as a percentage of the daily total of Co^{60} in the feces are tabulated directly below the bars to which they correspond. The scale for Fig. 2 is one-tenth that for Fig. 1. It is immediately apparent from the graphs that the fecal excretion of ingested Co^{60} reached a peak on the second day after dosing, as opposed to the first day after intravenous injection. This observation reflects the fact that most of the fecal Co^{60} following the injected dose probably entered the lower portion of the gastro-intestinal tract and hence was excreted faster than the oral Co^{60} which had to traverse the entire tract. Regarding the percentage composition of Co^{60} in the fecal fractions it can be calculated from Fig. 1 and 2 that over a 7-day period an average of 12% of the fecal Co^{60} from the orally dosed sheep was in the vit. B_{12} fraction, as opposed to 2% after injection. In absolute amounts, then, 10% of the orally administered inorganic Co^{60} appeared in the fecal B_{12} fraction, whereas only 0.2% of the injected dose was found in the feces as vit. B_{12}. These data substantiate the concept that appreciable amounts of vit. B_{12} can be synthesized from ingested cobalt, and since the intravenously injected cobalt apparently entered the alimentary tract below the site of most active B_{12} biosynthesis, it may be assumed most of the biosynthesis of vit. B_{12} occurs above the



PER CENT OF DAILY Co^{60}							
Total Co^{60}	100	100	100	100	100	100	100
Bound Co^{60}	83.4	79.0	79.0	82.9	80.4	82.4	82.0
Inorganic Co^{60}	15.4	18.2	18.1	14.5	18.0	15.8	16.0
Vit B_{12}-Like Co^{60}	1.2	2.8	2.9	2.6	1.6	1.8	2.0

FIG. 2. Average excretion of Co^{60} in feces of two sheep after intravenous injection plotted as % of dose/day; the tabulated values indicate the % of total Co^{60} represented by each fraction. The total fecal excretion for the week amounted to 8% of the dose.

duodenum, probably in the rumen.

It may be further noted in Fig. 1 that the absolute amount of cobalt-labeled B_{12} excreted in the feces was highest on the fourth day, while the peak excretion of total Co^{60} and the other fractions occurred on the second day. Moreover, the B_{12} fraction increased from 4% of the total Co^{60} excreted on the first day to 20% on the fourth day, after which time it remained essentially constant. These data, when considered in terms of the rate of movement in the tract, give a rough indication of the rate and degree of vit. B_{12} formation in the digestive tract. In Fig. 2, on the other hand, it is seen that after injection the fecal B_{12} fraction was remarkably constant at 2 to 3% of the total fecal Co^{60} except for the first day, when the value was low. Again, this observation probably indicates a time lag due to the formation of vit. B_{12} and subsequent movement down the tract. In this connection it should be noted that the fractional composition of the Co^{60} excreted every day by the injected sheep was very similar to that of the first day's feces from the animals dosed orally. This relationship may be taken to indicate that the ingested Co^{60} reaching the feces on the first day passed through the rumen too quickly to be acted upon appreciably by the organisms present, and hence behaved similarly to the Co^{60} reaching the tract after intravenous injection.

Urinary excretion. Values for the daily excretion of total, B_{12}-like, and inorganic Co^{60} in the urine are presented in Table I. Urine was found not to contain an insoluble bound fraction. In general, the total urinary Co^{60} following both routes of administration was highest on the first day and decreased steadily thereafter. Especially noteworthy was the large amount of the intravenous dose excreted in the urine on the first day, followed by a sharp drop on the second day. The percentage of the dose in both the B_{12} and inorganic fractions followed the same general pattern as the total Co^{60}, but it is important to note that the injected animals were excreting about 5 times as much radioactive vit. B_{12} as those dosed orally. However, as shown in the last 2 columns of Table I, the percentage of total

TABLE I. Urinary Excretion of Total Co^{60}, Inorganic Co^{60} and Vit. B_{12}-Like Co^{60} after Oral and Intravenous Administration of Co^{60} to Sheep.

	Total Co^{60}, % of dose		Inorganic Co^{60}, % of dose		Vit. B_{12}-like, % of dose		% of total Co^{60} in urine represented by vit. B_{12}	
Days	Oral	I.V.	Oral	I.V.	Oral	I.V.	Oral	I.V.
1	4	57.3	3.8	55.2	.21	2.1	5.2	3.7
2	3.7	6.3	3.5	5.6	.24	.62	6.5	9.9
3	1.5	6.8	1.4	6.3	.12	.51	8.2	7.6
4	.7	2.3	.6	2	.07	.27	9.9	11.9
5	.3	2.4	.3	2.1	.03	.31	8.7	13
6	.2	1.1	.2	.9	.02	.14	9.5	13.2
7	.4	1.5	.4	1.4	.02	.10	3.9	7
Total	10.8	77.7	10.2	73.5	.71	4.05	7.4*	9.5*

* Averages.

urinary Co^{60} made up by the B_{12} fraction was very nearly the same for both injected and orally dosed sheep, with the values for the injected animals tending to be slightly higher. These percentages differed from the general pattern of total Co^{60} excretion in the urine in that they were low on the first day, increased up to the 6th day, and had started to drop on the 7th day. This behavior probably is another manifestation of the rate of vit. B_{12} formation. Without doubt, the most significant import of these data is concerned with the source of the relatively large amounts of radioactive B_{12} in the urine of the intravenously injected sheep. Since it has been shown that little, if any, vit. B_{12} biosynthesis occurred in the gastrointestinal tract of the injected animals, it seems apparent that the peripheral tissues must have been responsible for some synthesis. The similarity of the values for oral and injected sheep in percentage of total Co^{60} as B_{12} can be interpreted as meaning that the cobalt in the blood and tissues was handled similarly in both cases. Furthermore, the small percentage of the ingested dose excreted as urinary B_{12} indicates that the biosynthesis which has been shown to take place in the tract of the orally dosed animals did not contribute significantly to the amount of vit. B_{12} in the urine, nor presumably to the amount reaching the tissues.

Tissue concentration. Fig. 3 presents values for the concentration of total Co^{60} and of the B_{12}-like, inorganic, and bound fractions in various tissues at the time of sacrifice. Average concentrations for each "oral" tissue are shown side by side with the concentrations of their "i.v." counterparts to facilitate comparison. As in Fig. 1 and 2, values for the percentage composition of Co^{60} in the tissue fractions are tabulated directly below the bar graph. The distribution of total cobalt is in general agreement with findings reported for other species(6-11). Following both modes of administration the highest concentrations of Co^{60} were found in the liver, kidney, adrenal, and pancreas. And, as would be expected from the excretion data, the concentrations of total Co^{60} were higher following an intravenous dose than after oral administration of the same amount of activity. In comparing the concentrations of Co^{60} fractions in the tissues it is noted that the concentration of the bound fraction was generally higher after an oral dose than after injection, whereas the reverse was true concerning inorganic Co^{60}. These observations probably are indications of the source of Co^{60} in each case, since all of the intravenous Co^{60} was injected as inorganic, whereas quite probably much of the ingested Co^{60} was already bound when absorbed. Obvious exceptions to this generalization, however, were the pancreas and spleen, in which there was little bound but much inorganic Co^{60} after either administration route. The adrenal glands, on the other hand, were noteworthy for the relatively high concentration of bound Co^{60} after intravenous injection.

Of particular importance is the fact that a higher tissue concentration of radioactive vit. B_{12} was obtained after intravenous injection of Co^{60} than after oral administration, even

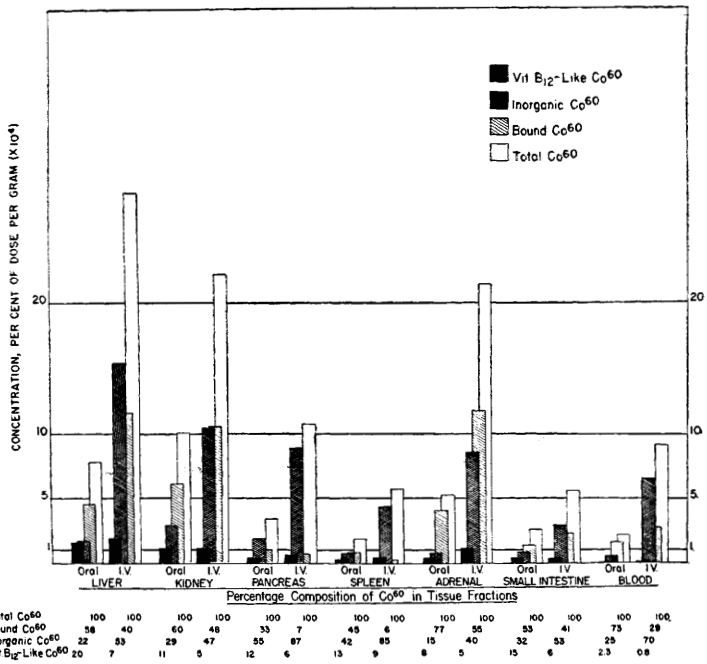


FIG. 3. Average concentrations of Co^{60} in different chemical fractions of various tissues of sheep after oral and intravenous administration. The tabulated values indicate % of total Co^{60} represented by each fraction.

though the vit. B_{12} fraction represented a smaller percentage of the total Co^{60} present in the former instance. Thus these data confirm decisively what the excretion data suggest, that some internal biosynthesis of vit. B_{12} must have taken place. The possibility that Co^{60} entered the B_{12} molecule by exchange seems unlikely in view of the failure of extensive efforts of Fantes *et al.*(17) to label B_{12} *in vitro* by exchange. Also, Chow *et al.*(14) found that no inorganic Co^{60} could be recovered in the urine of rats fed radioactive vit. B_{12}, indicating that no exchange took place *in vivo* under their conditions.

Indications regarding possible sites of B_{12} biosynthesis in the peripheral tissues may be obtained by presentation of the data in Fig. 3 in another way. A measure of the enrichment, or the degree of the accumulation, in a tissue may be obtained by calculating the ratio of the tissue B_{12}-Co^{60} to the blood B_{12}-Co^{60}. These ratios for several tissues are presented in the first 2 columns of Table II. When vit. B_{12} is introduced into the circulating blood one would expect a similar distribution pattern whether the B_{12} were introduced by direct injection or absorption from the tract. On the other hand, if vit. B_{12} were formed within a tissue from inorganic cobalt we might presume that this tissue would show a greater enrichment of the vitamin after injection than after oral administration of inorganic cobalt. By comparing the "i.v." enrichment against the "oral" enrichment after Co^{60} administration (column 3 of Table II), it is noted that only in the case of the adrenals and possibly the spleen was there a significantly greater enrichment of B_{12}-Co^{60} from the intravenously administered Co^{60}. This is considered to be presumptive evidence of vit. B_{12} biosynthesis in these tissues. As postulated, the ratios for the other tissues approach 1. The low value for the small intestine may be explained by the fact that in the orally dosed animals this tissue was continuously exposed to a considerable amount of B_{12} that had been synthesized in the tract.

The tissues were less efficient than the micro-organisms of the digestive tract in the biosynthesis of vit. B_{12} as can be shown by estimating the total amount of B_{12} formed in each case. On the basis of the B_{12}

TABLE II. Tissue Accumulation of Vit. B_{12}-Co^{60} from Blood after Oral and Intravenous Administration of Co^{60} to Sheep.

Tissue	Conc. of Vit. B_{12}-Co^{60} in tissue / Conc. of Vit. B_{12}-Co^{60} in blood		I.V.*
	Oral	I.V.	Oral
Liver	31.2	27	.87
Kidney	21.4	16.6	.78
Pancreas	8.2	9.6	1.17
Spleen	5	7	1.40
Adrenals	8.2	16.4	2
Small intestine	7.6	4.4	.58
Bone marrow	1.2	1.1	.92

* This represents the value in column 2 divided by that in column 1 (see text for explanation).

concentrations shown in Fig. 3, the average carcass concentration of B_{12} for the injected animals might be estimated liberally as 5 x 10^{-5}% of the dose per g. The total amount of B_{12} formed would then be (5 x 10^{-5}) (36,000 g body weight) = 1.5% of the dose, as compared with 10% of the dose found in the feces B_{12} of the orally dosed animals. It appears that at least 10 times as much of an oral dose of inorganic Co^{60} was converted to vit. B_{12} as was the case after intravenous injection. This is a conservative estimate and the true difference is probably much wider inasmuch as a liberal tissue value was used and the feces value is probably low, since no correction was made for degradation of the B_{12} in the tract. Barbee and Johnson(13) have shown that considerable degradation of vit. B_{12} occurs in the gastrointestinal tract of the rat.

Discussion. It is generally considered that cobalt is functional in all species in connection with its direct hematopoietic action. In ruminants there is an additional requirement which is concerned with the external function of cobalt in the rumen contents(18,19), which is probably related to bacterial action(20,21) and the production of B-vitamins(22,23). Becker and Smith(24) have shown that 1 mg of cobalt fed orally to cobalt deficient lambs will alleviate deficiency symptoms, whereas 1 mg of cobalt administered subcutaneously will not. On the basis of our results the tissues of their injected animals must have contained as much if not more vit. B_{12} than those of the orally supplemented lambs. This can only be interpreted to mean that either the vit. B_{12} as characterized in this paper was physiologically ineffective, at least insofar as gross symptoms of cobalt deficiency were concerned, or that the rumen requirements were not satisfied. Smith *et al.*(23) have recently reported that injection of 0.15 mg of a vit. B_{12} preparation produced a response in cobalt-deficient lambs. This would seem to indicate that the vit. B_{12} synthesized in the tissues from parenterally administered cobalt was physiologically ineffective, in contrast to that which reached the tissues after synthesis in the rumen.

Summary. 1. Excretions and tissues from wether lambs dosed orally and intravenously with inorganic cobalt-60 were measured for total radioactivity and also for radioactivity in fractions characterized chemically as vit. B_{12}-like, inorganic and bound. 2. After oral administration there was considerable biosynthesis of vit. B_{12} in the tract, probably occurring mainly in the rumen. After intravenous administration there was only slight, if any, biosynthesis in the tract, but considerable formation of vit. B_{12} in the tissues with the suggestion that the adrenals and spleen were mainly responsible. Overall, there was more than 10 times as much Co^{60} converted to B_{12} after ingestion than after intravenous administration. 3. The concentrations of vit. B_{12}-like Co^{60} in the tissues after intravenous injection were slightly higher than after ingestion of equal amounts of Co^{60}. 4. In the light of results on the response of cobalt-deficient lambs to parenterally administered vit. B_{12}, it is suggested that the vit. B_{12}-like material synthesized by the tissues from inorganic cobalt is physiologically ineffective in relieving cobalt-deficiency symptoms in sheep.

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Viability of the Lansing Strain of Poliomyelitis Virus in the Bat (*Eptesicus fuscus*). (19587)

REGINALD L. REAGAN, DOROTHY M. SCHENCK, AND A. L. BRUECKNER.

From the Live Stock Sanitary Service Laboratory, Md. State Board of Agriculture, University of Maryland, College Park.

The present study was undertaken in order to determine whether the bat (*Eptesicus fuscus*) might be a reservoir for poliomyelitis virus. Since under normal conditions the most probable method of exposure for the bat would be by the oral or intranasal routes, these 2 methods were used in the following experiment. As a control on the susceptibility of the bats, they were also inoculated by the intracerebral route.

Materials and methods. The Lansing strain of poliomyelitis virus was used. The strain was originally obtained by this laboratory in the form of mouse-brain suspension of the 200th mouse passage from Dr. David Bodian of the Poliomyelitis Research Center, Johns Hopkins University, Baltimore, Md. The virus had been passed 5 additional times in mice and once in cotton rats at the initiation of the present study. A 10% virus-bearing cotton-rat brain suspension was used as the inoculum for all bats. According to the Reed-Muench method of calculation(1) this material titrated $10^{-4.5}$ in cotton rats by intracerebral inoculation. The bats used in the present experiment were of the species *Eptesicus fuscus* and were in hibernation when they were obtained. They were kept kept in a heated room at the laboratory for 10 days before inoculation. During this period, all weak or inactive bats were discarded. The bats were divided into 3 groups of 28 bats each, and each group was exposed to the virus by the oral, intranasal, or intracerebral route. Bats of the intranasal and intracerebral groups were lightly anesthetized with ether before administration of the virus. The inoculum for each group was as follows: intracerebral, 0.03 ml per bat; intranasal, 0.02 ml per bat; and oral, 0.1 ml per bat. Following exposure to the virus, the bats of different groups were kept in separate cages in an isolation room with a lower temperature. Most of the bats went into hibernation and the remainder were very lethargic. On the 5th day post inoculation, 6 bats from each group were