encephalomyelitis (Theiler GD VII), 2 other APC strains (type 8 and strain DC), a Coxsackie virus, and influenza type B, produced no cytopathogenic effects in either passage, and except for a small amount of residual virus in the second passage of the APC type 8 and Coxsackie A9, there was no evidence of viral multiplication. In subsequent attempts with higher titer inocula, type 8 APC has been established in serial passage. It should be pointed out that the DC strain grows poorly in all cell types tested in tissue culture, including HeLa cells(7).

Summary. 1) A human epithelioid carcinoma cell in tissue culture (strain KB) has been found susceptible to a number of viruses, including poliomyelitis, herpes simplex, vaccinia, a number of APC strains, LCM and EMC. Marked cytopathogenic effects were produced in 1-7 days, and viral multiplication was shown by complement fixation, tissue culture, or animal titration of the second passage material. 2) Only partial and irregular cytopathogenic effects were obtained with mumps, yellow fever, and the SV_1 strain of APC virus, but with apparently continuing elaboration of virus on repeated passage. Both an egg-adapted and a mouse strain of mumps virus were found to have lost their infectivity for eggs after several culture passages. 3) Coxsackie virus (strain A9), influenza type B, one APC type, and GD VII had no cytopathogenic effect on this cell, and there was no evidence of viral multiplication. Rabies virus was cytopathogenic in the first passage only; and the harvest of the second passage was not infectious on intracerebral inoculation in mice.

1. Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 362.
2. ———, *J. Biol. Chem.*, 1955, v214, 839.
3. ———, *J. Exp. Med.*, 1955, v102, 37.
4. ———, *ibid.*, 1955, v102, 595.
5. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., *J. Biol. Chem.*, 1956, v218, 607.
6. Hull, R. N., Minner, J. R., and Smith, J. W., to be published.
7. Rowe, W. P., Hartley, J. W., and Huebner, R. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 260.
8. Jawetz, E., Kimura, S., Nicholas, A. N., Thygeson, P., and Hanna, L., *Science*, 1955, v122, 1190.

Received January 25, 1956. P.S.E.B.M., 1956, v91.

Comparative Absorption of Vitamin B_{12} Analogues by Normal Humans. II. Chloro-, Sulfato-, Nitro- and Thiocyanato- *vs* Cyanocobalamin. (22264)

CHARLES ROSENBLUM, RICHARD S. YAMAMATO, RUTH WOOD, DAVID T. WOODBURY, KUNIO OKUDA, AND BACON F. CHOW.

Merck Sharp & Dohme Research Laboratories, Rahway, N. J., and the School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.

The first(1) paper of this series reported results of comparative oral absorption studies of chlorocobalamin and cyanocobalamin employing the urinary excretion method of Schilling(2). These tests were interpreted as indicating the superiority of cyanocobalamin over chlorocobalamin for alimentary absorption by humans. Results of oral tolerance tests with humans, and of fecal excretion measurements with rats, confirming the low chlorocobalamin absorption, are reported herein. In addition, similarly inferior absorptions of sulfato-, nitro- and thiocyanatocobalamins are reported, based upon urinary and fecal excretion studies in humans, and upon fecal excretion by rats.

Methods. Materials. The preparation and properties of the radioactive and normal chlorocobalamins and cyanocobalamins employed in these experiments have been described(1). Sulfatocobalamins were obtained photochemically as for chlorocobalamin except that irradiation was performed in 0.01 M H_2SO_4 solution. Samples of the nitroco-

TABLE I. Urinary Excretion of Radioactivity by Humans.

Cobalamin			Avg % of dose
Oral		Inj., 1 mg	± stand. dev.
Cyano	2 μg	Cyano; chloro	≃12*
Sulfato	"	Sulfato	3.5 ± 1.5
		Chloro	2.6 ± .8
		Cyano	3.7 ± 1.8
Nitro	"	Nitro	2.0 ± .8
		Chloro	3.1 ± .5
		Cyano	1.9 ± .2
Thiocyanato	"	"	2.0 ± .9
Nitro	.5	"	5.8 ± 3.6
Cyano	"	"	17.6 ± 9.0

* Avg of accumulated standard test data.

balamins and thiocyanatocobalamins were prepared from sulfatocobalamins by treatment with acid nitrite and KSCN respectively. Paper chromatograms,* distribution coefficients and absorption spectra attested to the identity of the radioactive and normal forms. Specific activities of the radioactive cobalamins were: cyano-≃200 μc/mg; sulfato-≃130 μc/mg; nitro-≃130 μc/mg and thiocyanato-≃171 μc/mg. The urinary excretion(2,3) and the Heinle-Welch(4) fecal excretion (5-6 days) methods employed to compare the several labeled cobalamins have been adequately described elsewhere. In all cases the subjects (groups of 4-6) were young, healthy adult males under continual supervision to insure complete collection of excreta. Urine and feces radioactivity was determined by scintillation counting. Two series of oral tolerance tests were performed with chloro- and cyanocobalamin as described by Chow(5). Subjects were fed 3 or 5 mg of test substance in aqueous solution after first removing 15 ml of fasting blood for microbiological assay of serum for vitamin B_{12}. Two hours after ingestion, another 15 ml sample of blood (still fasting) was removed for vit. B_{12} assay. Aged subjects, *i.e.* over 65 years, were employed in order to reveal more readily resultant changes in serum vit. B_{12}. Only differences in excess of 40 μμg/ml are considered experimentally significant(5).

* The authors are grateful to R. D. Babson of the Research Laboratories of Merck & Co., for these chromatographic tests.

Normal adult male rats of the Johns Hopkins colony, weighing an average of 300 g per animal, were employed (in groups of 6) in fecal excretion experiments with cyano-, chloro- and nitrocobalamin-Co^{60}. A 50 mμg dose of labeled analogue was fed to each rat by stomach tube and feces collected for 5 days. All collections were measured for cobalt 60 content by scintillation counting of feces homogenates. Actually, excretion of radioactivity was negligible after the first 24-48 hours.

Results. Human urinary excretion results are compiled in Table I. Table II contains the results of 2 fecal excretion studies with 0.5 μg of cyano- and nitrocobalamin-Co^{60} administered to human subjects. Although daily measurements were performed, only cumulative excretions for the entire collection periods are of interest. Group average cumulative excretions measured, and average absorption values computed therefrom by difference, are reported.

Results of the 2 oral tolerance tests are compiled in Table III. Initial concentrations and maximum difference, observed after 2 hours, are reported in terms of μμg/ml of serum.

TABLE II. Fecal Excretion of Radioactivity by Humans.

Study	Cobalamin (.5 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
1st* (5 days)	Cyano	18.0 ± 7.1	82.0
	Nitro	41.8 ±12.8	58.2
2nd (6 days)	Cyano	13.7 ± 4.4	86.3
	Nitro	46.5 ± 6.9	53.5

* 1 mg vit. B_{12} injected 2 hr after oral dose of tracer.

TABLE III. Serum Levels (μμg/ml) in Humans.

	Cyano		Chloro	
Study	Initial	Difference (2 hr)	Initial	Difference (2 hr)
1st	280	682	222	0
3 mg cyano;	146	854	216	0
3 mg chloro	251	70	157	0
	169	286	642	0
	227	94	570	305
2nd	99	204	169	76
3 mg cyano;	122	158	140	41
5 " chloro	157	141	251	46
	227	94	490	0
	198	64	146	0

TABLE IV. Fecal Excretion of Radioactivity by Rats.

Cobalamin (.050 μg oral)	Avg % excr. ± stand. dev.	Avg % absorbed
Cyano	65.4 ± 7.2	34.6
Chloro	90.6 ± 9.5	9.4
Nitro	85.2 ± 15.2	14.8

Table IV is a summary of the findings from the fecal excretion study with cyano-, chloro- and nitro-cobalamin-Co^{60} administered to rats. Here too, group average cumulative excretions (after 5 days) and computed absorptions, are reported.

Discussion. Application of the urinary excretion method at 2 μg to sulfato-, thiocyanato- and nitrocobalamin reveals lower responses than are obtained with cyanocobalamin, as can be seen from the summary Table I. For nitrocobalamin, this is true also at the 0.5 μg oral level. The low sulfato-excretions (≃3% of the 2 μg dose) are practically identical with those reported previously for the chloro-analog, and are about one-fourth the average 12% figure typical of vit. B_{12} itself. Similar behavior characterizes nitrocobalamin and thiocyanato cobalamin *i.e.* low responses of 1.9-3.1% of the 2 μg dose independent of the choice of injected analogue. Even at 0.5 μg oral intake, the urinary nitrocobalamin output of 5.8% is much less than the 17.6% elimination of cyanocobalamin. Furthermore as in the case of labeled chloro- (1), the nature of the injected cobalamin is immaterial. Thus sulfato-, chloro-, nitro- and cyano-cobalamin are indistinguishable in their capacity to induce cobalamin excretion upon injection, which again points to the equivalence, perhaps through interconvertibility of cobalamins or conversion to another common structural analogue, of the several cobalamins once introduced into the blood stream.

The similarity in behavior of the chloro- and sulfato-forms is understandable in terms of their identical electrolyte and hydrophilic properties(6,7) and spectra. Less evident is the reason for the slight responses obtained with nitrocobalamin and thiocyanato-cobalamin. These compounds are characterized as electrically neutral cobalamins(7,8) since the substituent anions are relatively tightly bound(9) and because of their relative organophilic tendencies(9,10). In this respect they resemble cyanocobalamin rather than aquocobalamin salts, and might have been expected to exhibit a pronounced absorption closely approximating that of vit. B_{12} itself. Despite evidence for slight dissociability in certain cases, addition of cyanide ion converts all known cobalamins to the cyano form. The current results then point to the unique importance of the latter group in governing the metabolic activity of the cobalamins, rather than to the dissociability of the substituent anion. It is conceivable that even the low non-cyano responses are themselves due to partial interactions with traces of cyanide ion in gastric juice or upper intestinal contents rather than to an inherent activity of these cobalamins.

That the extent of urinary excretion after injection is an index of absorption has been verified by independent methods of demonstrating absorption.† In the case of chlorocobalamin, oral tolerance tests (Table III; first study) with humans failed to reveal any increase in serum cobalamin concentration in four out of five subjects fed 3 mg doses; and even feeding of 5 mg (Table III; second study) produces a barely significant rise in 3 out of 5 persons. By contrast, ten individuals receiving 3 mg of cyanocobalamin each exhibited serum levels 2 hours after ingestion which were on the average approximately 2-3 times normal. Further evidence for the superior absorption of cyanocobalamin is provided by the fecal excretion measurements with rats. It is obvious from Table IV that the absorption (9.4% of the 50 mμg oral dose) of chlorocobalamin is only about one-fourth the 34.6% absorption typifying cyanocobalamin.

Similar confirmation has been obtained in

† G. B. J. Glass, N. Y. Medical College has observed(11) by hepatic uptake method that the cpm of liver projections after oral administration of 0.5 μg of chlorocobalamin-Co^{60} is only one-half to one-third the radioactivity noted after administration of cyanocobalamin-Co^{60}. This is in accord with conclusions presented in our paper.

studies comparing the fecal excretion of labeled nitrocobalamin with that of cyanocobalamin by rats and humans. In the rat studies (Table IV) nitrocobalamin absorption (14.8%) is less than half the 34.6% value obtained with the cyano- form. In the first of the human studies (Table II), fecal excretions of nitro- and cyano- forms were respectively 41.8% and 18.0% of the 0.5 μg oral dose which presumably correspond to absorptions of 58.2% and 82.0%. Respective absorption figures of 53.5% and 86.3% obtained in the second study are in excellent agreement. When one considers that "zero" absorption values, as are obtained with pernicious anemia patients(12), may range from 0 to 24% (average≃12%), these figures would indicate a 2 to 1 superiority in absorption in favor of cyanocobalamin.

It is significant that all methods support the observations, based upon the urinary excretion method, that cyanocobalamin excels the other cobalamins as an oral absorption form. One may conclude further that, as regards the cobalamin family, the injection-excretion test can serve as an index by which to judge comparative absorption.

Summary. 1. Urinary excretion measurements indicate considerably lower oral absorption of sulfato-, nitro- and thiocyanatocobalamin than is exhibited by cyanocobalamin. In the blood stream, however, the several cobalamins are equivalent in inducing the excretion of ingested vit. B_{12}. 2. That cobalamin urinary excretion responses truly constitute an absorption index was verified in studies comparing chlorocobalamin with cyanocobalamin, by serum level assays in humans and by fecal excretion experiments with rats. 3. Inferior absorption of orally administered nitrocobalamin was confirmed by fecal excretion observations in humans and rats. 4. The superior absorption of cyanocobalamin (vit. B_{12}) over other cobalamins studied is attributed to either the chemical specificity of the substituent cyano group or the tightness of its binding in the cobalt coordination sphere.

1. Rosenblum, C., Woodbury, D. T., Gilbert, J. P., Okuda, K., and Chow, B. F., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 63.

2. Schilling, R. F., *J. Lab. Clin. Med.*, 1953, v42, 860.

3. Chow, B. F., Gilbert, J. P., Okuda, K., and Rosenblum, C., *Am. J. Clin. Nutrition,* in press.

4. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H., *Trans. Assn. Am. Physicians,* 1952, v65, 214.

5. Chow, B. F., *Symposium on Problems of Gerontology,* National Vitamin Foundation, New York, Aug. 1954, p59.

6. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *J. Am. Chem. Soc.*, 1951, v73, 3569.

7. Smith, E. L., Ball, S., and Ireland D., *Biochem. J.*, 1952, v52, 395.

8. Heinrich, H. C., and Lahann, H., *Zeits. Vitamin-, Hormon- Fermentforschung,* 1954, vVI, [No. 2-3], 126.

9. Smith, E. L., Fantes, K. H., Ball, S., Waller, J. G., Emery, W. B., Anslow, W. K., and Walker, A. D., *Biochem. J.*, 1952, v52, 384.

10. Buhs, R. P., Newstead, E. G., and Trenner, N. R., *Science,* 1951, v113, 625.

11. Glass, G. B. J., personal communication.

12. Callender, S. T., Turnbull, A., and Wakisaka, G., *Brit. Med. J.*, 1954, v1, (No. 4852), 10.

Received December 16, 1955. P.S.E.B.M., 1956, v91.