and excess acid chloride were then removed under reduced pressure, and the ester distilled *in vacuo*. A colorless or pale yellow liquid was obtained at 115–122° at 75 mm.; 6.70 g. (90.6%) was obtained. The product had a faint ester odor combined with a slight pungency which may have been due to retention of traces of the acid chloride.

Anal. Calcd. for $C_4H_6O_2FBr$: Br, 43.21; F, 10.27. Found: Br, 43.08; F, 9.86.

Fluoroacetylcholine Bromide.—A 15 × 120 mm. Pyrex test-tube was marked to contain 2.4 ml. (1.6 g., 0.027 mole) of trimethylamine. The amine was allowed to distil into the tube, which was cooled in an ice–salt-bath, until the mark was reached, when the tube was sealed off, a slender, fragile neck being left on the tube. This tube was then dried and placed inside a larger Pyrex tube which already contained 6.0 g. (0.032 mole) of β-bromoethyl fluoroacetate dissolved in 20 ml. of dry benzene. The large tube was then sealed off, and the small tube was broken inside the large one by careful manipulation. After 48 hours at room temperature the crystalline mass was collected and washed with two 20-ml. portions of boiling anhydrous acetone. The crystals (colorless needles) were quickly transferred to a vacuum desiccator for removal of the remaining acetone, after which a beaker containing P_2O_5 was placed in the desiccator. The compound melts at 124° (uncor.) and is very hygroscopic. 5.3 g. was obtained, 80.5%.

Fluoroacetylcholine bromide may be recrystallized from absolute alcohol. It gives the ester test of Hestrin.[6]

Anal. Calcd. for $C_7H_{15}O_2NFBr$: F, 7.78; Br, 30.5. Found: F, 7.50; Br, 30.6.

Trichloroacetylcholine Perchlorate.—13.95 g. (0.10 mole) of choline chloride was placed in a clean, dry erlenmeyer flask and 33.9 g. (0.12 mole) of trichloroacetyl chloride was added. The mixture was stirred by hand until it had become hard and appeared fairly dry. It was kept in an ice-bath all the while. The solid mass was dissolved in 20 ml. of ice-cold absolute ethanol and 15 ml. of 70% $HClO_4$ was added to this solution. A crystalline precipitate formed immediately. This was filtered off under suction and washed with cold absolute alcohol. The filtrate was placed in the ice-box and the precipitate (m.p. 184–192°) dissolved in anhydrous acetone and precipitated with dry ether. This raised its melting point to 192–197° (uncor.), which value remained constant upon further recrystallization. Meanwhile the filtrate deposited crystals of choline perchlorate (m.p. 273°).

Trichloroacetylcholine perchlorate is a colorless crystalline compound, solubility in water slightly less than 10 mg. per ml., hydrolyzed quite rapidly compared to acetylcholine, and undergoing very rapid alcoholysis in hot absolute alcohol. It is soluble in boiling acetone and may be recrystallized therefrom. It is not hygroscopic.

Anal. Calcd. for $C_7H_{13}O_6NCl_4$: Cl, 40.2; N, 4.01. Found: Cl, 40.6; N, 3.92.

Dichloroacetylcholine Perchlorate.—This was prepared by the method given above for trichloroacetylcholine perchlorate. The compound was obtained as colorless needles, m.p. 126° (uncor.), non-hygroscopic.

Anal. Calcd. for $C_7H_{14}O_6NCl_3$: Cl, 33.8; N, 4.45. Found: Cl, 34.0; N, 4.50.

Fluoroacetylcholine Perchlorate.—This was prepared from the bromide: colorless, non-hygroscopic needles, m.p. 89° (uncor.), insoluble in absolute alcohol, very soluble in acetone.

Anal. Calcd. for $C_7H_{15}O_6NFCl$: Cl, 13.5. Found: Cl, 13.7.

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The Exchange Stability of Cobalt in Vitamin B_{12}

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The characterization of vitamin B_{12} (cyanocobalamin) as a cobalt coördination complex[1] was based originally on the firmness of the cobalt binding, the apparent trivalency of this element in the compound, and its slight conductance in aqueous solution. This has been confirmed more recently by measurements of magnetic susceptibility.[2,3,4]

An indication as to the stability of the complex, and the nature of the bonds linking the complexed groups to the cobalt atom, can be obtained from the rate of exchange[5] of cobalt ions in a solution of vitamin B_{12} and radioactive cobaltous sulfate or vice versa. Fantes, Page, Parker and Smith[6] have observed no exchange of cobalt (from radioactive cobaltous sulfate) after 2 hours of admixture in 0.1 *N* acid or alkali, and 1 hour in neutral boiling aqueous solution. In view of the known slow deactivation of vitamin B_{12} under such conditions,[1,6] it is conceivable that, despite the negative results of the short time experiments, prolonged contact of vitamin and cobaltous salt would result in a detectable exchange of cobalt with the cobalt of the vitamin proper or in its gradually formed degradation products. We have, therefore, performed similar experiments in 0.01 *N* H_2SO_4, in 0.01 *N* NaOH and in distilled water (*p*H 5.5–6), but have extended the study for about three months at room temperature and for about two weeks at 55°.

As outlined above, the exchange experiments would fail to reveal irreversible decomposition of vitamin B_{12} with formation of ionic cobalt. This possibility was investigated by the use of radioactive (cobalt 60) vitamin B_{12}[7] mixed with normal cobaltous sulfate. A single experiment on the effect of light, which converts[8,9] vitamin B_{12} to other cobalamins,[10] was also carried out.

The exchange experiments were performed by adding a known amount of cobaltous sulfate (equivalent to 4.5–5 μg. cobalt) to an aqueous solution of vitamin B_{12} of equivalent cobalt content in ground glass-stoppered cylinders of Pyrex glass, and stored in the dark at room temperature (≃22°) and 55° for various periods of time. Parallel mixtures of cobaltous (60) sulfate[11] with non-radioactive vitamin B_{12}, and inactive cobalt with radioactive (Co^{60}) vitamin B_{12}, were prepared. Total volumes per experiment amounted to 11 ml., and total radioactivity was 3000–3400 c.p.m.; measured in 1 inch diameter stainless steel planchets with a thin window GM counter system with a 6% counting efficiency. Cylinders containing only the radioactive cobalt salt solution, or simply

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[11] Purchased from Tracerlab, Inc., on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

radioactive vitamin B_{12} solution, were also provided as blanks for detecting spurious effects such as adsorption, precipitation by alkali or incomplete extraction of cobalt.

The extent of exchange was determined by adding to the cylinders, after a fixed storage period, 10 ml. of carbon tetrachloride solution containing 1 mg. of purified dithizone,[12] shaking for ten minutes to extract the free cobaltous ion, separating the phases by centrifugation or standing for 20 minutes, and evaporating 2 ml. aliquots of each phase in 1 inch diameter stainless steel planchets under an infrared lamp. Acid or alkali solutions were carefully neutralized and the *p*H checked prior to extraction. Under the conditions obtaining in these experiments, the extraction efficiency exceeded 99% provided the *p*H was 4.9 or greater.

An attempt was also made to induce the exchange photochemically by irradiating 40 ml. of a 0.01 *N* HCl solution containing 1.51 mg. B_{12} and 24.2 μg. Co^{60} (as sulfate) with a 100-watt tungsten lamp at a distance of 3 inches for 17.5 hours with concomitant air aspiration. Under these conditions, the vitamin was completely converted[8,9] to other cobalamins as demonstrated spectrophotometrically.

Experiments with radioactive cobaltous sulfate are reported in Table I which gives per cent. of added radioactive cobalt left in the aqueous phase after treatment with dithizone. This represents the maximum amount of radioactive cobalt actually transferred to the vitamin. A "% Co^{60}" value of 50.8% would mean complete exchange (assuming 4.5% cobalt in vitamin B_{12}). Table II gives analogous results for the experiments involving radioactive vitamin, *i.e.*, the per cent. of extractable radioactive cobalt found in the CCl_4 phase. In this case a value of 52.6% would signify complete exchange. In the photochemical experiment, 0.24% of the radioactivity remained unextracted, which was identical with the value obtained in a blank irradiation experiment.

TABLE I

EXTRACTION OF COBALTOUS ION FROM AQUEOUS SOLUTIONS OF VITAMIN B_{12} AND $Co^{60}SO_4$

	Storage conditions		Co^{60} unextracted, %	
Temp., °C.	Medium	Days	Blank	Expt.
Room temp.	Water	3	0.33	0.96
		16	...	.69
		81	...	.0
		84	.76	...
	0.01 *N* H_2SO_4	16	.0	.14
		84	.20	.37
	0.01 *N* NaOH	16	.54	2.42
		84	56.1	51.3
55°	Water	1	0.78	0.4
		1	.21	0.01
		3	.59	2.58
		9	.43	0.37
		16	2.25	1.95
		16	42.9	16.3
		16	28.2	21.2
	0.01 *N* H_2SO_4	9	0.65	0.07
		16	1.22	0.43
	0.01 *N* NaOH	9	95.8	52.9
		16	97.0	96.0

TABLE II

EXTRACTION OF COBALTOUS ION FROM AQUEOUS SOLUTIONS OF $CoSO_4$ AND Co^{60} LABELLED VITAMIN B_{12}

	Storage conditions		Co^{60} extracted, %	
Temp., °C.	Medium	Days	Blank	Expt.
Room temp.	Water	0	...	0.28[a]
		84	0.21	.42
	0.01 *N* H_2SO_4	84	0.19	.37
	0.01 *N* NaOH	84	11.7	8.8
55°	Water	16	0.91	0.78
	0.01 *N* H_2SO_4	16	0.60	0.52
	0.01 *N* NaOH	1	3.2	1.0
		16	7.3	4.7

[a] Test of the effect of carrier cobalt on the extraction.

It is evident from Tables I and II that in no case is the extent of cobalt 60 transfer to an inactive component greater than in the corresponding blank run. Except for the experiments in alkaline medium and several of the 55° experiments in water, the "% Co^{60}" values which would signify exchange are small and variable, and differ but little from results noted in the absence of nonradioactive components. This probably represents incomplete separation of phases. One may conclude that by the method employed in this investigation no exchange can be detected after about three months of storage at room temperature in water and 0.01 *N* H_2SO_4.

High blank values in 0.01 *N* NaOH indicate that the apparent exchange observed in this medium is associated with the cobalt sulfate alone. These abnormal effects probably represent removal of cobalt ions either by adsorption on glass[13] or by formation of insoluble cobalt hydroxide.[14] The decrease in extractable cobalt after long standing in water at 55° may be due to slow leaching of alkali from the glass, leading to adsorption or precipitation. Significant blanks with radiovitamin in alkali probably result from alkaline degradation of vitamin B_{12} with liberation of cobalt ion, possibly followed by slow adsorption or precipitation, and is not surprising in view of the considerable decoloration which occurs simultaneously. Although the influence of alkali on free cobalt ion prevents an unequivocal conclusion, there is strong indication that B_{12} possesses considerable exchange stability even in alkaline medium.

One must conclude, from the absence of exchange, that the cobalt binding in vitamin B_{12} is of the covalent type rather than ionic. This is in accord with the diamagnetic nature[2,3,4] of the compound, and supports the supposition that trace impurities are responsible for the slight paramagnetism[3,4] exhibited by certain vitamin preparations. The failure of light to induce the cobalt exchange in B_{12} may indicate a similar type of cobalt binding in the cobalamins produced by irradiation, although the total contact period was less than one day.

RAHWAY, N. J.

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