STUDIES OF THE MECHANISM OF THE EFFECT OF HOG INTRINSIC FACTOR CONCENTRATE ON THE UPTAKE OF VITAMIN B_{12} BY RAT LIVER SLICES [1]

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An enhancement of the uptake of Co^{60}-labeled vitamin B_{12} by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC) was reported by Miller, Raney, and Hunter (1), and has been confirmed by Latner and Raine (2). This report is concerned with the mechanism of this enhancement. A preliminary report of this work has been presented (3).

MATERIALS AND METHODS

The basic protocol for each experiment was the following: a healthy adult male rat of the Sherman strain was sacrificed. His liver was immediately removed and placed in 0.9 per cent NaCl at 0° C. The liver was sliced with a Stadie-Riggs microtome designed to prepare slices 0.5 mm. thick. Each slice was immediately placed in a 20 ml. beaker containing 5 ml. of incubation medium and kept in a mixture of water and crushed ice at 0° C. The standard incubation medium was Hastings' bicarbonate buffered at pH 7.8 (4). The beaker and medium were weighed before and after addition of the slices, and the weight of the slices was calculated by difference. The weight of liver slices used was 200 to 300 mg. per incubation vessel.

Incubation medium was then added to each beaker to a final volume of medium in milliliters equal to 30 times the weight of the slices in grams (*i.e.*, when the slices were calculated to weigh 0.2 Gm., the volume of the medium was made up to 6 ml.).

A preparation of HIFC [3] was next added, contained in a volume of 0.9 per cent NaCl equal to 10 per cent of the final volume of the incubation medium (*i.e.*, when the final volume of the medium was 6 ml., the HIFC was added in 0.6 ml. of 0.9 per cent NaCl). Vitamin B_{12}-Co^{60} [4] (specific activity 1 μc. per μg.) was then added, also in a volume of 0.9 per cent NaCl equal to 10 per cent of the final volume of the incubation medium. Equivalent volumes of 0.9 per cent NaCl, without HIFC, but with vitamin B_{12}-Co^{60}, were added to control samples. The final concentration of vitamin B_{12}-Co^{60} was 830 $\mu\mu$g. (830×10^{-12} Gm.) and that of HIFC was 0.27 μg. per ml. This concentration of vitamin B_{12}-Co^{60} was chosen as a convenient compromise between the upper limits of the physiologic level of vitamin B_{12} in rat serum and the lower limits of adequate vitamin B_{12}-Co^{60} for accurate scintillation counting. At this concentration of vitamin B_{12}-Co^{60}, adsorption onto glass was very slight.

Incubation was performed in a Dubnoff metabolic shaker-incubator. The gas phase was 95 per cent O_2-5 per cent CO_2, shaking was at 92 cycles per minute, and the period of incubation was three hours.

At the termination of incubation, the supernatant solution was decanted and the slices were washed in 30 volumes of incubation medium at 3° C. After washing, the slices were incubated for five minutes in another 30 volumes of incubation medium at 37.5° C., the medium was decanted, and a final wash in 30 volumes of incubation medium at 3° C. was performed.

TABLE I

Uptake of vitamin B_{12}-Co^{60} by rat liver slices in three incubation media—hog intrinsic factor concentrate

Incubation medium		Counts per minute per gram of slices
Experiment A:		
Standard	Control	280
	HIFC*	2,020
Experiment B:		
Standard, with pyruvate added	Control	140
	HIFC	1,330
Krebs-Ringer phosphate	Control	220
	HIFC	750
	HIFC × 4.3	860
	HIFC × 0.05	220

* Hog intrinsic factor concentrate.

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[2] Senior Research Fellow, New York Heart Association and Heart Fund, Inc.

[3] No. WES655, kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, New York. This preparation was clinically active in a daily oral dose of 1 mg., as determined by urinary excretion studies of the Schilling type (5).

[4] Purchased from Merck, Sharpe & Dohme, Rahway, New Jersey, as a stock solution containing 1 μg. vitamin B_{12}-Co^{60} per ml., with a specific activity of 1 μc. per μg.

Radioactivity retained in the slices was determined in a well-type scintillation counter. Each sample was counted for a minimum of 10,000 counts. Radioactivity was expressed as counts per gram of wet liver slices per minute.

All experiments were done in duplicate. In each table, the counts of duplicate samples have been averaged and the average reported. Individual samples rarely exceeded a 5 per cent deviation from the average, except where total counts were less than 300.

RESULTS

Buffers

In standard medium, with or without added pyruvate (4), the uptake of vitamin B_{12}-Co^{60} by liver slices incubated with HIFC is as much as tenfold that observed in controls (Table I).

In Krebs-Ringer phosphate buffer at pH 7.2 (6), addition of HIFC resulted in uptakes of vitamin B_{12}-Co^{60} that were two to three times greater than controls (Table I). The effect of HIFC is not manifested at a 1 : 20 dilution and is increased only slightly, if at all, by a 4.3-fold concentration of HIFC.

The importance of buffer *per se* is open to question, since 0.9 per cent NaCl containing $CaCl_2$ was as good as the best buffer studied (Table II).

TABLE II

Effect of altering the ionic composition of the incubation medium on vitamin B_{12}-Co^{60} uptake of rat liver slices in the presence or absence of added hog intrinsic factor concentrate

Incubation medium		Counts per minute per gram of slices
Experiment F:		
Standard	Control	370
	HIFC*	2,620
Standard with bicarbonate replaced by chloride	Control	420
	HIFC	1,410
Standard with magnesium replaced by potassium	Control	440
	HIFC	2,640
Standard with calcium replaced by potassium	Control	390
	HIFC	40
Experiment G:		
Standard	Control	340
	HIFC	1,160
Standard with potassium replaced by sodium	Control	350
	HIFC	1,480
0.9% NaCl	Control	810
	HIFC	150
0.9% NaCl containing 10 mM $CaCl_2$	Control	400
	HIFC	1,260

* Hog intrinsic factor concentrate.

TABLE III

Effect of heating or 2,4-dinitrophenol on ability of rat liver slices to take up vitamin B_{12}-Co^{60} in the presence or absence of active or heat inactivated hog intrinsic factor concentrate

		Counts per minute per gram of slices
Experiment C:		
Standard experimental conditions	Control	250
	HIFC*	2,090
Slices preheated	Control	190
	HIFC	190
2,4-dinitrophenol added	Control	230
	HIFC	2,060
Experiment D:		
Standard experimental conditions	Control	560
	HIFC	1,790
	HIFC heated 10 min.	650
Experiment E:		
Incubation for 1 hour	Control	420
	HIFC	930
	HIFC heated 15 min.	430

Heating liver slices

Immersion of the beaker containing liver slices in a boiling water bath for 10 minutes prior to incubation had no measurable effect on the retained radioactivity of control liver slices (Table III). However, such heating abolished the enhancement of vitamin B_{12}-Co^{60} uptake associated with the addition of HIFC.

Heating HIFC

The addition of HIFC which had been heated in a water bath at 100° C. resulted in slight, if any, enhancement of uptake of vitamin B_{12}-C^{60} (Table III). The differences in increase of uptake with unheated HIFC in experiments C and D are due to variation from rat to rat.

2,4-dinitrophenol

Addition of 2,4-dinitrophenol in a concentration of 10^{-4}M did not diminish the influence of HIFC on the uptake of vitamin B_{12}-Co^{60} (Table III).

Time of incubation

In the presence of HIFC, the rise in vitamin B_{12}-Co^{60} uptake throughout the first hour appears

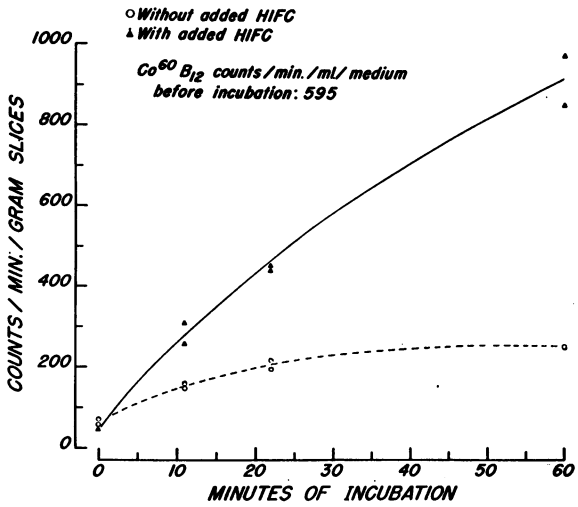

FIG. 1. EFFECT OF TIME OF INCUBATION ON UPTAKE

to be quite rapid, with decrease in uptake per unit time appearing subsequently (Figures 1 and 2).

Temperature

It is noteworthy that the effect of HIFC in enhancing the uptake of vitamin B_{12}-Co^{60} is demonstrable at 3° C. as well as at 37.5° C. (Figure 2).

Ionic composition of incubation medium

Each of the ions of the standard medium, with the exception of chloride, was replaced in turn with an equivalent millimolar concentration of another ion present in the incubation medium (Table II).

No significant effect on vitamin B_{12}-Co^{60} uptake was observed in control slices regardless of the ion replaced. The enhancing effect of HIFC was demonstrable in the absence of any one ion *except calcium.* In the absence of calcium, HIFC actually decreases the uptake of vitamin B_{12}-Co^{60}. Unpublished observations indicate the explanation of this phenomenon is that the vitamin B_{12}-Co^{60} is all bound to HIFC, and therefore unavailable to the liver slices except in the presence of calcium.

Incubation of liver slices in 0.9 per cent NaCl with and without added calcium results in uptakes of vitamin B_{12}-Co^{60} similar to those observed in the standard medium with and without calcium (Table II).

The uptake of vitamin B_{12}-Co^{60} in the presence of added HIFC was greater at a concentration of 10 mM calcium than at 1 mM. At 0.1 mM, there was no enhancing effect on addition of HIFC (Table IV).

The possibility was investigated that a divalent ion close to calcium in the periodic table might substitute for calcium. Strontium was found to be an effective substitute; other ions studied were not. These included: magnesium, cobalt, barium, cadmuim, mercury, tin, zinc, and beryllium. For these studies Krebs-Ringer solution buffered at pH 7.5 with 0.1 M trishydroxy-amino-methane buffer (7) was used. In this buffer, 10 mM concentrations of all the ions tested could be reached, (except in the cases of barium, tin, and zinc, where somewhat lower concentrations had to be used).

Effect of ethylenediaminetetraacetate (EDTA)

Liver slices were incubated for one hour and then washed in the standard manner. Aliquots were incubated for a second hour in 20 volumes of standard buffer, to which was added 2 volumes of 2 Gm. per cent disodium ethylenediaminetetraacetate dihydrate (EDTA) or 2 volumes of 0.9 per cent NaCl.

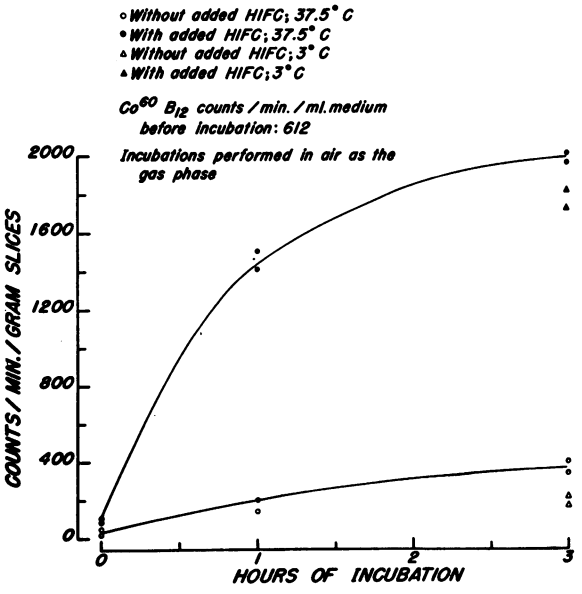

FIG. 2. EFFECT OF TIME AND TEMPERATURE OF INCUBATION ON UPTAKE

As noted in Table V, the addition of EDTA did not bring about any greater loss of vitamin B_{12}-Co^{60} from controls than did the addition of 0.9 per cent NaCl. However, the addition of EDTA markedly reduced the amount of vitamin B_{12}-Co^{60} retained in liver slices previously incubated in the presence of added HIFC.

Heparin, chondroitin sulfate, and orosomucoid

No increase in vitamin B_{12}-Co^{60} uptake by rat liver slices was demonstrated on addition to the incubation medium of heparin, chondroitin sulfate,[5] or orosomucoid,[6] in concentrations of 0.26 or of 8.3 μg. per ml. of incubation medium.

DISCUSSION

The studies here reported demonstrate certain aspects of the enhancement of the uptake of vitamin B_{12}-Co^{60} by rat liver slices in the presence of HIFC. The enhancement occurs in the cold as well as at 37.5° C. It is observable only in the presence of calcium (or strontium) in adequate concentration. It does not occur if either the liver slices or the HIFC are heated prior to incubation. The presence of 2,4-dinitrophenol in 10^{-4} M concentration does not diminish the effect of HIFC. The major portion of the vitamin B_{12}-Co^{60} taken up by rat liver slices in the presence of HIFC may be subsequently removed by incubation in the presence of EDTA.

TABLE IV

Effect of varying the calcium concentration of the incubation medium on the vitamin B_{12}-Co^{60} uptake of rat liver slices in the presence or absence of added hog intrinsic factor concentrate

Calcium concentration (*mM*)		Counts per minute per gram of slices
10.0	Control	860
	HIFC*	3,170
1.0	Control	680
	HIFC	1,880
0.1	Control	630
	HIFC	570
0.01	Control	630
	HIFC	470

* Hog intrinsic factor concentrate.

[5] Kindly provided by Dr. D. Hamerman.

[6] Kindly provided by Dr. G. B. J. Glass, from a lot prepared by Dr. R. J. Winzler.

TABLE V

Effect of ethylenediaminetetraacetate on retention by rat liver slices of vitamin B_{12}-Co^{60} taken up previously in the presence or absence of added hog intrinsic factor concentrate

Second incubation		Counts per minute per gram of slices
Not performed	Control	490
	HIFC*	1,210
Performed, no EDTA† added	Control	290
	HIFC	900
Performed, EDTA added	Control	290
	HIFC	360

* Hog intrinsic factor concentrate.
† Ethylenediaminetetraacetate

These data suggest that the mechanism whereby HIFC enhances vitamin B_{12}-Co^{60} uptake by rat liver slices is physical rather than metabolic. Should this interpretation be correct, the possibility would remain that vitamin B_{12}-Co^{60} held to the liver slice by HIFC might subsequently be acted on by metabolic processes within the cell.

Heparin, chondroitin sulfate, and orosomucoid do not increase vitamin B_{12}-Co^{60} uptake by rat liver slices. This would indicate that enhancement of vitamin B_{12} uptake is not a nonspecific property of any mucopolysaccharide or mucoprotein.

SUMMARY

Vitamin B_{12} uptake by rat liver slices is enhanced markedly in the presence of hog intrinsic factor concentrate. This enhancement is observable at 37.5° C., at 3° C., and in the presence of 10^{-4}M 2,4-dinitrophenol. It requires calcium (or strontium), and is reversible to an appreciable extent by EDTA.

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