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Stoichiometric Relation between Liver-Receptor, Intrinsic Factor and Vitamin B_{12}.* (25765)

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Since the original report that hog intrinsic factor concentrate (HIFC) enhanced Co^{60}-labeled Vit. B_{12} uptake by rat liver slices(1), the mechanism of this phenomenon has been extensively studied. Data obtained confirm the hypothesis that receptors for intrinsic factor exist on rat liver slices and the low speed precipitate of the homogenate thereof. These receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached(2). Identical receptors for intrinsic factor on small intestinal mucosa of the rat have also been demonstrated(3). The present report extends these studies and confirms the stoichiometry implied previously (2,4).

Materials and methods. Liver homogenate was prepared by mincing fresh (or fresh frozen) liver with scissors, adding 40 ml of 0.9% NaCl per 10 g liver, and homogenizing for 1 minute at room temperature in a Waring blendor. The homogenate was centrifuged at 3000 rpm for 5 minutes, the supernate discarded, and the remaining homogenate washed twice in 0.9% NaCl (40 ml/10 g initial liver tissue). The washed homogenate of "liver debris" (mainly cell walls and nuclei) was suspended in Krebs-Ringer-Tris containing 10 mM $CaCl_2$ (KRT) or in 0.9% NaCl containing 10 mM $CaCl_2$(NaCl-$CaCl_2$); 10 ml of medium was used per 1 g of whole liver starting material. Although it was generally used immediately, washed homogenate prior to suspension in medium may be refrigerated for as long as 2 hours prior to use if necessary. Incubations were performed for one half hour, in air, at room temperature, with constant gentle shaking. Each incubation beaker contained the washed homogenate from 1 g of liver, or 100 mg of lyophilized liver homogenate, suspended in 10 ml of medium, plus 1 ml of the appropriate added agent. Each incubation was followed by centrifugation at 3000 rpm for 5 minutes, discarding the supernatant fluid by decantation, and 3 washes of the homogenate with 10 ml aliquots of NaCl-$CaCl_2$. Each incubation was preceded by suspending the washed homogenate in 10 ml incubation medium. Although subsequent incubations were usually performed immediately, the homogenate may be refrigerated for as long as 1 hour prior to subsequent incubation without loss of activity. One ml of each agent was added to the liver homogenate suspension. Solid materials were made up to 1 ml in 0.9% NaCl. The HIFC preparation used‡ was potent by Schilling-type testing(6) in a daily oral dose of 5 mg. Unless otherwise specified, 25 μg HIFC was used. The amount of Co^{60}-B_{12} added to each sample was 0.01 μg (10^{-8} g) (specific activ-

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TABLE I. Radioactivity Uptake by Homogenate of Frozen Rat, Bull, Cow, and Rabbit Liver after Sequential Incubation in HIFC and Co^{60}-B_{12}.

Homogenate source		Counts/min.
Rat liver stored 7 days at −20°C	Control	14 ± 1
	HIFC	1397 ± 67
Bull liver stored 1 day at −20°C	Control	43 ± 1
	HIFC	1016 ± 40
Cow liver stored 1 day at −20°C	Control	77 ± 8
	HIFC	643 ± 62
Rabbit liver stored 4 days at −20°C	Control	38 ± 8
	HIFC	2184 ±165
Rat liver homogenate precipitate stored 26 days at −20°C	Control	56 ± 7
	HIFC	870 ± 43
Rat liver homogenate suspended in KRT, stored 5 days at −20°C	Control	56 ± 7
	HIFC	145 ± 1

ity = 1 μc/μg). The well type sodium iodide crystal used had a sensitivity of 1 count/min/$\mu\mu$c. *Sequential* incubation (*i.e.*, incubation with HIFC followed by incubation with Co^{60}-B_{12}) was generally performed for reasons stated elsewhere(2,5). After final incubation, retained radioactivity of thrice-washed homogenate was determined, and results recorded as counts per minute per g of initial liver tissue (or per 100 mg of lyophilized liver homogenate). Lyophilized rat liver homogenate was prepared from liver minced with scissors, homogenized in 0.9% NaCl, washed thrice in 0.9% NaCl, and suspended in 0.9% NaCl (or NaCl-$CaCl_2$ if preincubated with HIFC) prior to lyophilization. About 5 g of lyophilized homogenate was derived from 100 g of liver tissue. Acetone-washed lyophilized liver homogenate was prepared by grinding lyophilized homogenate in a mortar with aliquots of acetone until supernate was clear. The final acetone-homogenate mixture was filtered and homogenate remaining on the filter paper was allowed to stand at 4°C until dry. Resultant material was stored at 4°C until used. Rat stomach homogenate was prepared as follows: the frozen stomachs of 10 rats, with the intrinsic factor-free proximal translucent portions discarded(7), were homogenized in 50 ml 0.9% NaCl in a Waring blendor at 6°C for 2 minutes, and filtered with suction (at 6°C) through gauze to remove particles. Fifteen ml 0.9% NaCl were mixed with the particles on the gauze, filtered, and added to the prior filtrate which was then frozen until used. pH of this material was 6.4.

Results. Table I demonstrates that rat, cow, bull, or rabbit liver frozen whole, or rat liver frozen *as the precipitate* of a homogenate, may subsequently be used as a source of receptors.

Rat liver homogenate frozen *while suspended in KRT* appears to lose most of its receptors (Table I). The receptors are protected to a significant degree by incubation with HIFC prior to freezing (Table II).

Lyophilized or lyophilized and acetone-washed rat liver homogenate retains its ability to be used as a source of receptors (Table III). The receptor-intrinsic factor complex may be lyophilized as a unit and retain its ability to "take up" Co^{60}-B_{12} (Table II).

The receptor-intrinsic factor-Co^{60}-B_{12} complex requires calcium for maintenance of its integrity (Tables III, IV). Calcium appears to maintain the receptor-intrinsic factor bond rather than the intrinsic factor-Co^{60}-B_{12} bond (Table IV).

Quantity of HIFC required to "saturate" homogenate receptors from 1 g of fresh rat or frozen cow liver was about 50 μg, as indicated by failure of amounts greater than 50 μg to still further enhance Co^{60}-B_{12} uptake by the homogenate (Table V). Amounts of HIFC much in excess of that required to saturate the receptors actually produced less enhancement than smaller quantities (Table V).

TABLE II. Co^{60}-B_{12} Uptake by Rat Liver Homogenate Incubated with HIFC, then Stored Prior to Addition of Co^{60}-B_{12}.

Conditions of storage	Time stored (days)	Counts/min.
Exp. A:		
Suspended in KRT at −20°C	1	2347 ± 236
Idem	7	2390 ± 0
"	28	2310 ± 54
Exp. B:		
Not stored	0	4746 ± 0
Suspended in KRT at −20°C	3	1663 ± 16
Exp. C:		
Lyophilized; 5°C	7	1708 ± 116
Idem	28	1975 ± 158

TABLE III. HIFC Effect on Co^{60}-B_{12} Uptake by Fresh, Lyophilized, or Lyophilized and Acetone-Washed Rat Liver Homogenate; Similar in KRT or NaCl-$CaCl_2$ Medium.

Medium	Homogenate	Agent added before first incubation	Counts/min.
KRT	Fresh	NaCl	73 ± 4
"	"	HIFC (.5 mg)	6588 ± 45
NaCl-$CaCl_2$	"	NaCl	85 ± 2
"	"	HIFC (")	7058 ± 75
KRT	Lyophilized*	NaCl	28
"	"	HIFC (")	6533
NaCl-$CaCl_2$	"	NaCl	37
"	"	HIFC (")	5973
KRT	Lyophil.-acet.†	HIFC (.25 mg)	2811 ±251

* 100 mg, stored 4 days at 5°C prior to use. † 100 mg, stored 4 days at 5°C after lyophilization and acetone-washing.

The intrinsic factor-receptor complex is destroyed by heating at 100°C for 10 minutes prior to addition of Co^{60}-B_{12}, although HIFC itself retained about 25% activity after such heating (Table VI).

TABLE IV. Calcium Dependence of HIFC Effect.

1st incubation Medium	1st incubation Added agent	2nd incubation Medium	Counts/min.
Exp. A (fresh liver homogenate)			
NaCl	NaCl	NaCl	58 ± 1
	HIFC	"	159 ± 30
NaCl-$CaCl_2$	NaCl	NaCl-$CaCl_2$	102 ± 86
	HIFC	"	3196 ±445
	NaCl	NaCl	44 ± 4
	HIFC	"	70 ± 1
NaCl	NaCl	NaCl-$CaCl_2$	34 ± 4
	HIFC	"	104 ± 37
Exp. B (lyophilized liver homogenate)			
KRT	HIFC	KRT	2049
KRT-Ca*	"	"	19

* Calcium replaced by equivalent millimolar concentration of sodium.

Rat stomach homogenate, pH 6.4, enhanced Co^{60}-B_{12} uptake by rat liver homogenate (Table VI), but its effect was reduced by standing for 30 minutes at pH 2.6. The stomach homogenate after standing 2 hours at 3°C at pH 6.4, retained much, but not all, of its original activity (Table VI).

TABLE V. Effect of Varying HIFC Concentration on Co^{60}-B_{12} Uptake by Fresh Rat and Frozen Cow Liver Homogenate.

Agent added prior to 1st incubation	Rat liver Counts/min.	Cow liver Counts/min.
NaCl	56 ± 7	77 ± 8
25 μg HIFC	1264 ± 171	643 ± 62
50 μg "	3994 ± 14	1084 ± 93
100 μg "	4021 ± 41	973 ± 78
500 μg "	3410 ± 200	211 ± 34

TABLE VI. Depression of HIFC and Rat Stomach Homogenate Effects by Heat or Low pH.

	Agent added prior to 1st incubation	Counts/min.
Exp. A	NaCl	94 ± 5
	HIFC	1835 ± 52
	" *	58 ± 3
	" heated 10 min. at 100°C	517 ± 52
Exp. B	NaCl	33 ± 2
	HIFC	1740 ± 30
	RSH†	865 ± 37
	" †, heated 30 min. at 37°C, pH 2.6	341 ± 6
	" †, after 2 hr at 3°C	659 ± 35

* Prior to 2nd incubation, homogenate heated 10 min. at 100°C.
† Rat stomach homogenate.

Gradual reduction of amount of Co^{60}-B_{12} added prior to the second incubation resulted in a gradual increase in percentage of Co^{60}-B_{12} remaining on the homogenate "receptor-intrinsic factor complex" after incubation (Table VII).

By varying the quantities of HIFC and of Co^{60}-B_{12} within the limits imposed by the quantity of receptors on liver homogenate, a point is approached where a stoichiometric relationship exists between these quantities (Table VIII).

When a mixture of Co^{60}-B_{12} and non-radioactive B_{12} (cold B_{12}) is added to liver homogenate preincubated with HIFC, a reduction of Co^{60}-B_{12} uptake is observed which is proportional to amount of "cold" B_{12} used (Table IX).

Discussion. The present demonstration

TABLE VII. Co^{60}-B_{12} Uptake by Rat Liver Homogenate, Using Sequential Incubation and Decreasing Amounts of Co^{60}-B_{12}.

Co^{60}-B_{12} added prior to 2nd incubation ($\mu\mu$g)	Counts/min.
9660	2308 ±20
4830	1753 ± 3
2415	1205 ± 1
966	720 ± 9
97	92 ± 2

that the "intrinsic factor receptors" on mammalian liver remain intact after lyophilization and acetone extraction of the liver indicate the possibility of further purification of these receptors, with the ultimate goal of a pure system of receptors, intrinsic factor, and B_{12}. If there is a stoichiometric relationship between these 3, as the present data indicate, it becomes possible to assay the amount of any one of these substances by determining the others.

Until intrinsic factor is isolated in pure form, the nature of the material combining with the receptors will remain uncertain. The activity may be due to a contaminant common to HIFC, rat stomach homogenate, and normal human gastric juice(2). Similarly, degraded intrinsic factor or other materials with the appropriate prosthetic group(s) may attach to receptors and block uptake of intrinsic factor(8,9), and intrinsic factor to which "cold" B_{12} is bound cannot be assayed in this system(8). Excessive amounts of intrinsic factor must be avoided to prevent erroneous results due to loose attachment of excess HIFC to the homogenate. Some of this may not be removed in the washes and be released into the incubation medium during subsequent incubation with Co^{60}-B_{12} to compete with intrinsic factor on receptors for available B_{12}

TABLE VIII. Effect of Varying Quantities of HIFC and of Co^{60}-B_{12} in Sequential Incubation System with Rat Liver Homogenate.

HIFC (μg)	Co^{60}-B_{12} ($\mu\mu$g)	Counts/min.
0	2898	14 ± 1
20	2898	888 ± 93
20	1932	746 ± 79
20	966	437 ± 74
25	2898	1208 ± 8
25	1932	1074 ± 13
25	966	591 ± 44
30	2898	1533 ± 82
30	1932	1102 ± 45
30	966	592 ± 6

(2). Optimum amount of intrinsic factor to use for intrinsic factor assay would be that quantity sufficient to enhance Co^{60}-B_{12} uptake markedly, but not to saturate receptors.

Apropos of the possible relation of the present system to the physiologic system for absorption of B_{12} from the human small intestine(2), it should be noted that in the human small intestine there is also a stoichiometric relation between intrinsic factor and absorption of B_{12}(10).

TABLE IX. Reduction of Radioactivity Uptake by Rat Liver Homogenate in Sequential Incubation System When Cold B_{12} Mixed with Co^{60}-B_{12}.

Agent(s) added before incubation: 1st incubation	2nd incubation	Counts/min.
Exp. A:		
NaCl	Co^{60}-B_{12} + NaCl	20 ± 1
HIFC	" + "	1117 ± 17
	" + 10,000 $\mu\mu$g B_{12}	432 ± 6
	" + 5,000	612 ± 19
	" + 2,500	768 ± 1
	" + 1,000	932 ± 16
Exp. B		
NaCl	Co^{60}-B_{12} + NaCl*	27 ± 1
HIFC	" + NaCl*	1097 ± 27
	" + 5,000 $\mu\mu$g B_{12}*	660 ± 52
	" + 2,500*	771 ± 1
	" + 1,250*	895 ± 8
	" + 500*	966 ± 20
	" + 250*	1019 ± 9
	50*	1028 ± 37

Co^{60}-B_{12} used was one-half ml, specific activity 1 μc/μg; 4,465 $\mu\mu$g/½ ml.

* In 5 ml of 0.9% NaCl.

Rat intrinsic factor (rat stomach homogenate) is capable of marked enhancement of Co^{60}-B_{12} uptake by rat liver homogenate in the *sequential* incubation system here used. This latter point is important in view of the failure of homologous gastric juice to enhance Co^{60}-B_{12} uptake by rat liver slices in a *simultaneous* incubation system(11,2).

Summary. "Receptors" for intrinsic factor exist in rat, bull, and rabbit liver homogenate precipitate, and remain intact after lyophilization and acetone-washing of the homogenate. There appears to be a stoichiometric relation between receptors, intrinsic factor, and Vit. B_{12}. Within stated limits, an incubation system of receptors, intrinsic factor, and/or Co^{60}-B_{12} may prove useful for assay of intrinsic

factor, of the quantity of free B_{12} in biological fluids, and of receptors.

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Removal of Chylomicron Lipid from Plasma in Experimental Nephrosis.* (25766)

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Hyperlipemia is a characteristic feature of experimental nephrosis produced in rats by injection of rabbit anti-rat kidney serum. One factor contributing to the hyperlipemia is a decrease in rate of removal of chylomicron lipid from the circulation(1,2). Two possible explanations for this abnormality are suggested. First, the delayed removal may be dependent on presence in nephrotic plasma of an inhibitor to lipoprotein lipase which is responsible for intravascular lipolysis of chylomicrons(3). Second, the suggestion has been made that nephrotic hyperlipemia is due to retention of lipid in the plasma as the result of hypoalbuminemia, on the assumption that the amount of serum albumin is insufficient to transfer fatty acids at a normal rate from lipoproteins to the tissues(4). The following studies were undertaken to assess the effects of serum albumin concentration and of lipoprotein lipase inhibitor on rate of chylomicron removal from the circulation of rats made nephrotic by antikidney serum.

Methods. Nephrosis was produced in rats by injection of rabbit anti-rat kidney serum (5). Rat serum albumin was obtained from normal rat serum after precipitation of globulins by third-saturated ammonium sulfate. It was dialyzed against normal saline to remove excess sulfate, lyophilized, and made up in concentrated solution for administration. On electrophoretic examination the albumin was 87% pure. For *in vitro* studies on clearing of lipemic serum, the source of lipoprotein lipase was post-heparin plasma, obtained from the aorta of fasted normal rats 15 minutes after intravenous injection of heparin (4 mg/kg body weight). Post-heparin plasma was incubated with normal rat serum previously made lipemic by addition of rat chyle sufficient to give an optical density (O.D.) of 0.450-0.700 when read at 550 mμ or 750 mμ. In a preliminary experiment the O. D. of lipemic serum was found to be a linear function of amount of rat chyle added to it. To measure rate of removal of chylomicrons from the circulation, 1.0 ml of C^{14}-labeled chyle, prepared according to French and Morris(6), was injected intravenously; radioactivity was measured in whole blood at intervals over the next 30 minutes, by plating aliquots of 0.05 ml of tail vein blood and counting in a windowless proportional gas flow counter. The fractional rate constant of chylomicron re-

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