9. Overman, J. R., Tamm, I., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 806.

10. Sharp, D. G., *IV Internat. Kong. Electron mikros.*, 1958, v2, 542.

11. Bennett, H. S., *J. Biophys. Biochem. Cytol.*, 1956, v2, (Part 2), 99.

12. Sbarra, A. J., Karnofsky, M. L., *J. Biol. Chem.*, 1959, v234, 1355.

13. Backus, R. C., Williams, R. C., *J. Appl. Physiol.*, 1950, v21, 11.

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Studies Suggesting the Presence of Intrinsic Factor in Bile.* (26789)

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Evidence has previously been presented suggesting the presence of a "circulating intrinsic factor" involved in selective deposition of B_{12} in the liver(1). The present report exhibits 4 lines of evidence suggesting that intrinsic factor may be present in bile.

Materials and methods. Human bile was collected *via* a mushroom catheter in the gall bladder of a patient with complete occlusion of the common duct by a carcinoma of the head of the pancreas. Rat bile was obtained by means of catheterization,† under temporary anesthesia, of the bile duct of rats thereafter maintained for 6 to 24 hours in a special restraining cage with access to food and water.

Hog intrinsic factor concentrate (HIFC), WES 671 A, and purified HIFC, were obtained from the same source.‡ Their activity in man had been demonstrated by a modified Schilling test(2) showing a standard effect in pernicious anemia with single oral doses of 5 mg and of 0.3 mg, respectively. The Co^{60}-B_{12} used was of specific activity approximately 1 $\mu c/\mu g$.§

The liver homogenate system used has previously been described(3); each of the serial incubations was for ½ hour, in air, at room temperature. Everted sacs of rat small intestine were prepared and incubated during procedures carried out essentially as described by others(4). In all experiments a minimum of 10,000 counts were obtained on each sample. Samples which were less than twice background were counted for 10,000 counts on each of 3 separate occasions to ensure validity.

Antiserum to purified HIFC was prepared by injecting into each footpad of a rabbit 0.5 ml of 0.2 mg/ml purified HIFC weekly for a month, and subsequently harvesting serum at intervals separated by further injections of antigen. Agar double diffusion analysis was performed as described by Ouchterlony (5).

Results. Experiment 1, Table I, indicates that human bile increases the enhancing effect of HIFC on Co^{60}-B_{12} uptake by rat liver homogenate in a sequential incubation system. Exp. 2, shows that human bile by itself enhances Co^{60}-B_{12} uptake by rat liver homogenate. Exp. 3, parts a. and b., suggests that the effect of human bile, like that of HIFC(6), is calcium-dependent; part c. demonstrates that the enhancing effect of human bile following incubation is not due to physical entrapment of Co^{60}-B_{12}-laden bile in the liver homogenate. Thus without incubation liver homogenate showed little more uptake from Co^{60}-B_{12}-laden bile than from NaCl-$CaCl_2$.

Table II indicates that, like HIFC, human

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TABLE I. Enhancement of Frozen, Thawed Liver Homogenate Uptake of Co^{60}-B_{12} by HIFC and by Human Bile.

	1st incubation		2nd incubation		
Exp.	Medium	Added agents	Medium	Added agent	Counts/min.
1	$NaCl$-$CaCl_2$	NaCl	$NaCl$-$CaCl_2$	Co^{60}-B_{12}	66
	"	HIFC*	"	"	3318
	"	Human bile + HIFC*	"	"	3806
2	KRT,† pH 7.4	NaCl	KRT,† pH 7.4	"	105
	"	HIFC*	"	"	3371
	"	Human bile	"	"	529
	"	Serum from same human	"	"	129
3	(a) $NaCl$-$CaCl_2$	NaCl	$NaCl$-$CaCl_2$	"	167
	"	HIFC*	"	"	3000
	"	Human bile	"	"	845
	(b) NaCl	NaCl	"	"	263
	"	HIFC*	"	"	302
	"	Human bile	"	"	387
	(c) Human bile + Co^{60}-B_{12} incubated together for 1 hr, without added medium; then added to liver homogenate in $NaCl$-$CaCl_2$, stirred, and homogenate immediately precipitated by centrifugation, washed, then counted				189

* 1/200 daily oral dose of WES671A (25 μg).
† Krebs-Ringer tris, pH 7.4, containing 10 mM $CaCl_2$.

bile will enhance Co^{60}-B_{12} uptake by liver homogenate in a simultaneous incubation system. Furthermore, the HIFC-like action of human bile appears to be calcium-dependent and EDTA-reversible as is the effect of HIFC(6). Table III displays the ability of rabbit antiserum to purified HIFC to eliminate the enhancing effect of both HIFC and human bile. This table also indicates a slight HIFC-like effect of rat bile. Because rat bile showed only this slight intrinsic factor-like activity in the rat liver homogenate system, it was tested in the sensitive *in vitro* everted gut sac system used by Strauss and Wilson(4). Table IV demonstrates that in this system, some samples of rat bile had an intrinsic factor-like activity similar to that of rat stomach homogenate. This was not true for all rat biles tested, however. The concentrations of rat stomach selected for study were based on studies(7) which showed that either much less *or much more* rat stomach homogenate was inactive in the everted sac system.

In Table V, it may be seen that rat bile from each of 2 different donor rats (No. 1

TABLE II. HIFC-like Properties of Human Bile in Rat Liver Homogenate System: Effectiveness in Simultaneous (Bile + Co^{60}-B_{12}) Incubation; Calcium-dependence; EDTA-reversibility.

1st incubation		2nd incubation		3rd incubation		
Medium	Added agents†	Medium	Added agent†	Medium	Added agent†	Counts/min.
$NaCl$-$CaCl_2$	NaCl + Co^{60}-B_{12}	—	—	—	—	176
"	HIFC* + Co^{60}-B_{12}	—	—	—	—	3178
"	Human bile + Co^{60}-B_{12}	—	—	—	—	573
"	NaCl	$NaCl$-$CaCl_2$	Co^{60}-B_{12}	$NaCl$-$CaCl_2$	—	93
"	HIFC*	"	"	"	—	3748
"	Human bile	"	"	"	—	545
"	NaCl	"	"	"	2 g % EDTA	76
"	HIFC*	"	"	"	"	185
"	Human bile	"	"	"	"	222
NaCl	NaCl	"	"	"	—	86
"	HIFC*	"	"	"	—	103
"	Human bile	"	"	"	—	152

* 25 μg. † 1 ml.

TABLE III. Effect of Rabbit Antiserum to Purified HIFC on Enhancing Action of HIFC and of Human Bile on Co^{60}-B_{12} Uptake by Rat Liver Homogenate. Slight effect of rat bile.

Exp.	1st incubation*	2nd incubation*	3rd incubation*	Counts/min.
a.	NaCl + Co^{60}-B_{12}			132
	Rat bile #1 + Co^{60}-B_{12}			162
	" " #2 + "			191
	HIFC + "			2779
	HIFC† + "			2951
	Human bile + "			318
	(HIFC + rabbit antiserum)† + Co^{60}-B_{12}			51
	(Human bile + rabbit antiserum)† + Co^{60}-B_{12}			80
b.	HIFC	Rabbit antiserum	Co^{60}-B_{12}	674
	Rabbit antiserum	HIFC	"	2081
	HIFC	NaCl	"	3639
	NaCl	HIFC	"	2872

* All incubations were performed in Krebs-Ringer tris (KRT) medium, pH 7.4.
† Preincubated for 1 hr at 37°C before adding to liver homogenate.

and No. 3) appeared to enhance Co^{60}-B_{12} absorption by a gastrectomized rat to the same extent as did the homogenate of ½ rat stomach. However, bile from 2 other donor rats (No. 2 and No. 4) had no such effect.

Ouchterlony agar gel diffusion analysis showed (Fig. 1) a continuous precipitin band (reaction of identity) produced against purified HIFC and rat stomach homogenate by rabbit antiserum against HIFC, with a partial reaction of identity against human stomach homogenate. Similar diffusion analysis utilizing the same antibody demonstrated that a common precipitin line formed with human bile, human stomach homogenate and HIFC.

Discussion. The studies reported here suggest that intrinsic factor is present in bile. Thus, human, and to a lesser extent, rat, bile has HIFC-like activity in the rat liver homogenate semiquantitative "assay" for intrinsic factor. The activity of human bile is calcium-dependent and EDTA-reversible, as is the effect of HIFC. Some samples of rat bile appear to function like rat intrinsic factor in the rat small intestine everted sac qualitative "assay" for rat intrinsic factor. Human bile and intrinsic factor concentrates from 3 species (hog, rat, man) contain a related antigen, demonstrated by agar gel diffusion studies with an antiserum to purified HIFC. Finally, bile from 2 of 4 donor rats appears to possess intrinsic factor activity in a gastrectomized rat.

TABLE IV. Substitution of Rat Bile for Rat Stomach Homogenate in Everted Sacs from Middle of Rat Small Intestine.

Added agent	Counts/min. per 2 inch sac	Enhancement of B_{12} uptake (% above control)
None (control)	23	0
Homogenate of 1/100 rat stomach	113	491
.1 ml rat bile #1	60	261
" " " #2	85	369
" " " #3	44	192

Krebs-Henseleit bicarbonate incubation medium.

TABLE V. Enhancement of Co^{60}-B_{12} Absorption in Gastrectomized Rat Produced by Rat Bile.

Orally administered agents	% of administered Co^{60}-B_{12} in 7-day stool collection
Co^{60}-B_{12}* + 2 ml H_2O	97.4
Idem + ½ rat stomach (homogenate)	77.0
" + 2.2 ml rat #1 bile	76.6
" + 2 ml rat #2 bile	94.3
" + ½ rat stomach (homogenate)	76.1
" + 2 ml rat #3 bile	77.0
" + 2 ml rat #4 bile	95.0
" + 2 ml rat #4 bile	93.8

* 15,000 $\mu\mu$g in 1.5 ml 0.9% NaCl.

While these 4 separate lines of evidence are consistent with the conclusion that intrinsic factor is present in bile, the following equivocations must be introduced: 1. No *in vitro* test can be proved to assay intrinsic factor at the present time, since intrinsic factor has not as yet been isolated in pure form. Thus, both the liver homogenate and everted sac systems may actually measure a contaminant

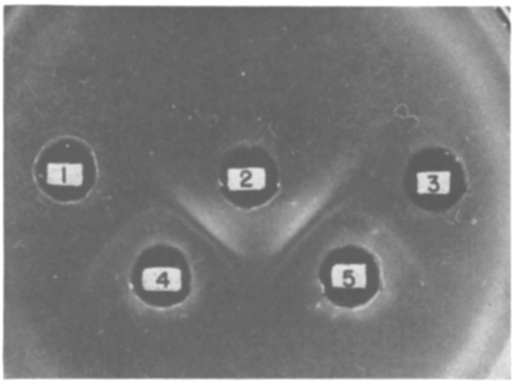

FIG. 1. Ouchterlony plate showing common precipitin line between rabbit anti-hog intrinsic factor and intrinsic factor concentrates from hog, rat, and human sources. 1. Rat stomach homogenate. 2. Purified hog I. F. 3. Human stomach homogenate. 4. Rabbit anti-hog I. F. 5. Rabbit anti-hog I. F.

present in all intrinsic factor concentrates so far prepared, rather than intrinsic factor itself. This possibility cannot be overlooked even though studies so far have shown that besides intrinsic factor only serum(1) and bile produce an enhanced Co^{60}-B_{12} uptake by rat liver systems; other agents either have no effect or many *reduce* the effect of HIFC (8). 2. The present definition of intrinsic factor activity requires that rat bile be repeatedly demonstrated to substitute for rat intrinsic factor in gastrectomized rats before it can be accepted to contain intrinsic factor activity. 3. The immunologic evidence that bile and intrinsic factor concentrates, even from different species, seem to possess a common antigen does not necessarily mean that the common antigen is intrinsic factor.

The results of the semi-quantitative liver homogenate "assay" suggest that amounts of human bile in excess of 1 liter would be necessary to produce an intrinsic factor effect in man, if in fact such an effect could be produced. No enhancement of Co^{60}-B_{12} uptake was obtained in Schilling(2) tests of 2 pernicious anemia patients and of one man who had undergone total gastrectomy when they were fed Co^{60}-B_{12} plus approximately 200 ml of human bile(9). Since this amount of bile is somewhat nauseating, it may be necessary to concentrate the "intrinsic factor activity" of much larger quantities of bile before feeding it or supplying it through a nasogastric tube.

The concept that bile contains intrinsic factor would fit well with the concept of a "circulating intrinsic factor"(1) involved in selective deposition of Vit. B_{12} in liver, and with the concept of the enterohepatic circulation of Vit. B_{12}(11-13) dependent upon or associated with an enterohepatic circulation of intrinsic factor. Toporek found that bile from rat liver which had been perfused with HIFC enhanced Co^{60}-B_{12} uptake in a second perfused rat liver(14), as did HIFC mixed with Co^{60}-B_{12}(15). While this later finding (14) may only mean that a complex of B_{12} bound to non-intrinsic factor B_{12}-binding substance in bile(11) was taken up by Kupffer cells as a foreign material(16), it could mean that intrinsic factor did in fact traverse the hepatic cells, as Toporek suggests. It may be possible to judge between these possibilities, and others, when his study is reported in full.

In observations aimed at narrowing down the possible sources of human intrinsic factor, Castle, Townsend and Heath(10), using a triple-lumen tube during occlusion of the pylorus by a balloon, secured bile-containing duodenal contents. On feeding approximately 75 cc of this material daily for 10 days to a patient with untreated pernicious anemia, they observed a reticulocyte peak of 4.8% (on day 8); whereas in a succeeding period, 75 cc of gastric juice caused a reticulocyte peak of 28.4% (on day 8). The conclusion was drawn that there was no intrinsic factor in bile and that the initial slight hematopoietic effect might have been due to traces of gastric juice entering the duodenum. Now, after a lapse of 3 decades, the possibility that, in the presence of a normally functioning stomach, bile may contain intrinsic factor is again raised. Indeed, intrinsic factor in liver cells or bile might explain the recent finding of Reizenstein and Nyberg(17) that intestinal absorption of liver-bound B_{12} exceeds that of an equivalent amount of B_{12} alone.

The failure of Co^{60}-B_{12} to be absorbed in man(18) and in the rat(19) after total gastrectomy suggests that, if bile does contain

intrinsic factor, its site of origin is the stomach. If absorbed from the intestine after binding Vit. B_{12}, its presence in bile would argue strongly for an entero-hepatic circulation of intrinsic factor in addition to those intestinal phases previously reviewed(20). Consequently, an important question to be answered is whether gastric intrinsic factor is absorbed directly into the blood stream like uropepsin from the cells which make it or is discharged into the stomach and then absorbed with Vit. B_{12} from the ileum after passage into the small intestine.

There are a number of possible explanations for our failure to find intrinsic factor activity in all specimens of rat bile, in studies depending on ability of intrinsic factor to bind radioactive B_{12}. They include: 1) Intrinsic factor may not be secreted at a constant rate in rat bile (too little or too much would be ineffective); 2) The considerable non-radioactive B_{12} content of bile(11-13) may intermittently be great enough to saturate all the intrinsic factor present. (We find an average of 3,000 to 5,000 $\mu\mu$g B_{12} normally present in 1 ml of rat bile by *Euglena gracilis*(21) assay.) Indeed, an unsuspected large isotope dilution effect may explain the apparent inhibition of B_{12} uptake by rat bile reported by others(22,23), whose analyses revealed only 3 $\mu\mu$g B_{12} per ml rat bile; 3) There may not be intrinsic factor in bile.

Summary. Four lines of evidence are presented suggesting that rat and human bile may contain intrinsic factor: 1. Human, and to a lesser extent rat, bile enhances Co^{60}-B_{12} uptake by rat liver homogenate. 2. Some rat bile specimens enhance Co^{60}-B_{12} uptake by everted sacs of rat small intestine. 3. Some rat bile specimens appear to substitute for rat intrinsic factor in a gastrectomized rat. 4. In Ouchterlony agar double diffusion analysis a reaction of identity was formed by human bile and intrinsic factor concentrates from hog, rat, and human sources to antibody induced by injection of purified hog intrinsic factor concentrate. If these findings in fact indicate that bile contains intrinsic factor, it is possible that an enterohepatic circulation of intrinsic factor exists as a further phase of its activity with relation to Vit. B_{12}.

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1. Herbert, V., Spaet, T. H., *Am. J. Physiol.*, 1958, v195, 194.
2. Ellenbogen, L., Williams, W. L., Rabiner, S. F., Lichtman, H. C., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 357.
3. Herbert, V., Castro, Z., Wasserman, L. R., *ibid.*, 1960, v104, 160.
4. Strauss, E. W., Wilson, T. H., *Am. J. Physiol.*, 1960, v198, 103.
5. Ouchterlony, O., *Acta path. microbiol. Scandinav.*, 1953, v32, 231.
6. Herbert, V., *J. Clin. Invest.*, 1958, v37, 646.
7. Herbert, V., Castle, W. B., *ibid.*, 1961, v40, November.
8. Castro, Z., Herbert, V., Wasserman, L. R., *ibid.*, 1961, v40, 66.
9. Herbert, V., Zalusky, R., Kaplan, M. E., unpublished data.
10. Castle, W. B., Townsend, W. C., Heath, C. W., *Am. J. Med. Sci.*, 1930, v180, 305.
11. Okuda, K., Gräsbeck, R., Chow, B. F., *J. Lab. Clin. Med.*, 1958, v51, 17.
12. Gräsbeck, R., Nyberg, W., Reizenstein, P. G., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 81.
13. Reizenstein, P. G., *ibid.*, 1959, v101, 703.
14. Toporek, M., *Fed. Proc.*, 1961, v20, 450.
15. ———, *Am. J. Physiol.*, 1961, v200, 557.
16. Herbert, V., *Fed. Proc.*, 1960, v19, 884.
17. Reizenstein, P. G., Nyberg, W., *Lancet*, 1959, v2, 248.
18. Paulsen, M., Conley, C. L., Gladsden, E. S., *Am. J. Med. Sci.*, 1950, v220, 310.
19. Nieweg, H. O., Arends, A., Mandema, E., Castle, W. B., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 328.
20. Cooper, B. A., Castle, W. B., *J. Clin. Invest.*, 1960, v39, 199.
21. Ross, G. I. M., *J. Clin. Path.*, 1952, v5, 250.
22. Honska, W. L., Driscoll, T. B., Johnson, P. C., *Clin. Res.*, 1960, v8, 201.
23. Johnson, P. C., Driscoll, T. B., Honska, W. L., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 181.

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