similar to those found in rats kept on the corresponding diets although the Vit. B_{12} organ levels in the latter were much lower(5).

The present results indicate that the relation between Vit. B_{12} and reduced liver glutathione is not a simple one and that the effect of the latter on the former is only evident under special experimental conditions.

Summary. No differences were found in liver soluble GSH levels between Vit. B_{12}-deficient and control mice, kept on otherwise normal diets, or on protein-free, or methionine-low rations supplemented with sulfur amino acids. When kept for a short period on diets free of protein, or low in methionine, or fasted, mice deficient in Vit. B_{12} had lower hepatic GSH levels than the corresponding controls. After 1 month on a methionine low diet, these levels were higher in B_{12}-deficient animals than in the corresponding controls. Cysteine, cystine, and methionine were equally effective in maintaining the soluble liver GSH at high levels in B_{12}-deficient or supplemented mice consuming protein-free diets.

The collaboration of Miss C. Embden and Miss N. Franceschi D. in glutathione determination is appreciated.

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Development of a Possible *in vitro* Assay for Intrinsic Factor.*† (23840)

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Miller, Raney, and Hunter(1,2) reported an enhancement of Co^{60}-labeled Vit. B_{12} uptake by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC), and this finding has been confirmed by Latner and Raine(3), and Herbert and London(4). The latter workers(4,5) reported that enhancement of Co^{60}-B_{12} uptake by HIFC was calcium-dependent, reversible to an appreciable degree by ethylene-diamine-tetraacetate (disodium Versenate®), and occurred in the cold as well as at 37.5°C. Most enhancement by HIFC occurred in first hour of incubation(5). Further studies on the mechanism of the enhanced uptake of Co^{60}-B_{12} by rat liver slices in the presence of HIFC are here reported, along with the development of a possible *in vitro* assay for intrinsic factor

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based on these studies. A preliminary report of this work has been presented(6).

Materials and methods. The incubation medium was Krebs-Ringer solution(7), maintained at pH 7.5 with 0.1 M tris-hydroxy-amino-methane (Tris) buffer(8), and with sufficient added $CaCl_2$ to bring the calcium concentration to 10 mM. Tris buffer was chosen because it does not limit the calcium concentration to the same extent as do phosphate and bicarbonate buffers. Two to three hundred mg of rat liver slices were placed in 20 ml beakers, each containing 10 ml of buffer. All incubations were performed for one hour, at 0°C, in air, with constant gentle shaking. Since the uptake of Co^{60}-B_{12} by rat liver slices without HIFC is lower in the cold than at 37°C(5), cold incubation produces a more striking demonstration of enhanced uptake in the presence of HIFC. On incubation at 37°C, liver slices become increasingly friable with time. Increased friability is accompanied by decreased reproducibility of results. No such increased friability is observed at 0°C. Each incubation was followed by decanting the supernatant fluid and washing slices 3 times in 10 ml aliquots of buffer. Where HIFC or Co^{60}-B_{12} was added prior to incubation, the added agent was first dissolved in one ml of 0.9% NaCl. Control samples had one ml of 0.9% NaCl added. The amount of Co^{60}-B_{12} added was always 0.01 μg (specific activity 1 μcurie/μg). For experiments with *simultaneous* incubation in HIFC and Co^{60}-B_{12}, both agents were added prior to a single incubation. For experiments with *sequential* incubation, only HIFC was added prior to first one hour incubation, and only Co^{60}-B_{12} prior to second one hour incubation. For all experiments, retained radio-activity of washed slices was determined in a well-type scintillation counter. The results were recorded in terms of Co^{60}-B_{12} counts/minute/g of liver slices. All HIFC preparations used were from the same source.§

TABLE I. Co^{60}-B_{12} Uptake by Rat Liver Slices after Simultaneous Incubation in HIFC and Co^{60}-B_{12}.

HIFC preparation	HIFC conc. (μg/11 ml)	Liver slice uptake (counts/min./g)
671A	500	495
	375	661
	250	994
	125	1090
	50	1109
	37.5	1535
	25	1795
	12.5	1257
	5	698
671B	500	684
	375	816
	250	1381
	125	1407
	50	1897
	37.5	1530
	25	1176
	12.5	755
	5	442
None	0	425
	0	363

TABLE II. Co^{60}-B_{12} Uptake by Rat Liver Slices after Simultaneous or Sequential Incubation in 500 μg of HIFC #671B.

1st hr		2nd hr		Liver slice uptake (counts/min./g)
HIFC	Co^{60}-B_{12}	HIFC	Co^{60}-B_{12}	
0	0	0	+	241
+	0	0	+	4900
+	+	*	*	644
+	+	0	+	5510
0	+	0	+	399
0	0	+	+	691
+	0	+	+	595

* No second hr incubation.

Results. Table I demonstrates that for any given preparation of HIFC there was an optimal concentration which produced maximal Co^{60}-B_{12} uptake by rat liver slices. Either increasing or decreasing the HIFC concentration from this optimal level resulted in a decreased enhancing effect on Co^{60}-B_{12} uptake.

Table II demonstrates that rat liver slices incubated in a high concentration of HIFC, washed, and subsequently incubated in Co^{60}-B_{12} take up much more Co^{60}-B_{12} than slices incubated in these materials together. Furthermore, slices which have taken up less Co^{60}-B_{12} because of being incubated in HIFC and Co^{60}-B_{12} *simultaneously* may have their Co^{60}-B_{12} uptake increased to that of *sequentially* incubated slices by undergoing a second incubation in Co^{60}-B_{12} alone.

Table III shows the results of ***in vitro*** assay of 7 HIFC preparations, as performed by

§ Kindly provided by Drs. W. L. Williams and L. Ellenbogen of Lederle Laboratories, Pearl River, N. Y.

TABLE III. Relative Potencies of 7 HIFC Preparations by *In Vitro* and *In Vivo* Determination.

Preparation	*In vitro*, counts/min./g*	*In vivo*, units of potency
WES655	4676,5095	50
671A	1522,1345	10
671C	1147,1316	7–10
671B	784,874	2
186–1	213,192	1
671F	30,54	½
671E	−18,15	0

* After subtraction of control values, which averaged 415 counts/min./g.

In vitro determinations were done in duplicate, and both results recorded.

the sequential incubation method. Fifty mg aliquots of each HIFC preparation were used. *Sequential* rather than *simultaneous* incubation was chosen for the assay procedure for reasons to be discussed. Table III also gives the *in vivo* abilities of the same 7 preparations to enhance radioactive-cobalt-labeled B_{12} absorption from the gastrointestinal tract of pernicious anemia patients, measured by a modification(9) of the Schilling urinary excretion test(10). "*In vivo* units of potency" are the calculated relative potencies of the various HIFC preparations, with the frequently studied(9,11,12,13) Lederle fraction #186-1 arbitrarily assigned a value of one unit. It is evident that the 2 methods of HIFC assay show good agreement as to relative activity.

Table IV shows the effect of one ml aliquots of gastric juice from 4 patients with histamine-fast achlorhydria and Vit. B_{12} deficiency disease. As would be expected if the *sequential* incubation method were an assay for intrinsic factor, the gastric juice from the 2 pernicious anemia patients had no effect on Co^{60}-B_{12} uptake by rat liver slices, but the gastric juice from the patients with nutritional B_{12} deficiency and malabsorption syndrome markedly enhanced Co^{60}-B_{12} uptake.

Table IV also demonstrates that 1 mg aliquots of heparin and chondroitin sulfate have no effect on Co^{60}-B_{12} uptake by rat liver slices in the *sequential* incubation method. Since both these agents bind B_{12}(14), clearly the *sequential* incubation method is not a measure only of B_{12} binding by intrinsic factor.

Discussion. The fact that there was an optimal concentration of HIFC producing maximal Co^{60}-B_{12} uptake by rat liver slices suggested a working hypothesis that receptors for intrinsic factor exist on the rat liver slice, and that in the presence of calcium these receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached. This hypothesis was supported by the observation that liver slices incubated in a high concentration of HIFC, washed, and subsequently incubated in Co^{60}-B_{12} would take up much more Co^{60}-B_{12} than slices incubated in these agents together. Two other possibilities suggest themselves to explain the experimental results. They are that an inhibitor of intrinsic factor action may be present in HIFC, or that non-intrinsic factor substances may be present in HIFC which compete with intrinsic factor for available Co^{60}-B_{12}. Any of these 3 possibilities can prevent enhancement of liver slice Co^{60}-B_{12} uptake in the presence of large amounts of HIFC. It was for this reason that *sequential* incubation in HIFC and then in Co^{60}-B_{12} was selected as the assay procedure.

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. The *sequential* incubation system has accurately reflected the *in vivo* potency of the HIFC preparations tested; it has accurately predicted the diagnosis of B_{12} deficiency due to lack of intrinsic factor (Addisonian pernicious anemia), when the differential diagnosis rested between Addisonian pernicious anemia and B_{12} deficiency on a basis other

TABLE IV. Sequential Incubation Assay of Gastric Juice from Patients with Vit. B_{12} Deficiency Diseases. Assay of heparin and chondroitin sulfate.

Assayed material	Liver slice uptake (counts/min./g) Controls	Assayed material
Gastric juice from patient with:		
Pernicious anemia	387, 342	397, 419
Idem	" "	370, 328
Nutritional B_{12} lack	298, 364	776
Malabsorption	404, 354	855, 859
Heparin	" "	411, 436
Chondroitin sulfate	" "	377, 376

All assays in duplicate; both results recorded.

than lack of intrinsic factor; and it has been demonstrated not to be a measure of B_{12} binding alone. These results would suggest that this system may be indeed an *in vitro* assay for intrinsic factor.

Summary. When rat liver slices are incubated in hog intrinsic factor concentrate (HIFC) and Co^{60}-B_{12} simultaneously, there is an optimal concentration of HIFC producing maximal Co^{60}-B_{12} uptake by the liver slices. Any deviation from this optimal level results in a decreased enhancement of Co^{60}-B_{12} uptake. Slices incubated in high concentration of HIFC, washed, and then incubated in Co^{60}-B_{12} take up much more Co^{60}-B_{12} than slices incubated in these materials together. From these facts the hypothesis was derived that receptors for intrinsic factor may exist on rat liver slices, and that these receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached. A possible *in vitro* assay for intrinsic factor is presented, based on sequential incubation of rat liver slices in intrinsic factor-containing buffer and then in Co^{60}-B_{12}.

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Effect of Chromatotrophic Hormone on Pigments of Anuran Skin.* (23841)

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Recently, it has been demonstrated that both guanophores and melanophores of anurans are under the influence of the chromatotrophic hormone, CTH(1). Furthermore the relative amount of guanine contained in the guanophore appeared to be governed by CTH. These results were questionable, however, in the light of Gunder's report(2) which stated that the term guanophore was a misnomer and that this chromatophore actually contains no guanine. In an attempt to clarify this problem, the present investigation was undertaken to determine whether guanine is indeed the pigment-like substance of the guanophore and furthermore, whether CTH restricts the amount of this purine in these cells.

Materials and methods. The present experiments were performed on normal and hypophysioprivic larvae of *Rana pipiens* and *Rana sylvatica*. Eggs of *Rana pipiens* were obtained by induced ovulation and those of *Rana sylvatica* were collected near Rochester, N. Y. A few adult *Rana pipiens* were also used. The hypophyseal placode was removed from embryos at an early tailbud stage and about 30 hypophysioprivic and partially hypophysioprivic larvae were selected for the ex-

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