In vitro Assay of Hog Intrinsic Factor with Rat Liver Homogenates. (24151)

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In this paper we describe an *in vitro* assay for hog intrinsic factor which has proven to be very useful. The method is based upon earlier work of Miller(1) and others(2-4) in which they showed that intrinsic factor could increase the Vit. B_{12} uptake of rat liver slices. In our method liver homogenates are used, since the results are similar to those with slices but a much greater precision can be obtained. Certain intrinsic factor preparations were found to cause a lower Vit. B_{12} uptake than expected in this *in vitro* assay, indicating presence of an inhibitor. The relationship between this inhibitor and an inhibitory intrinsic factor similar to that reported by Tauber and co-authors(5) is under investigation.

Materials and methods. Male Sprague-Dawley rats were used which weighed 200-350 g. They had been fed a standard high-vitamin casein diet including 100 μg Vit. B_{12}/kg diet. Animals were decapitated and bled before hepatectomy. Liver homogenates (1 g liver/5 ml buffer) were prepared in loose-fitting Teflon and glass homogenizer in ice and were strained through cheesecloth. The buffer was at pH 7.1 and had the following composition in mM/liter: K, 135; Mg, 25; Ca, 12.5; Cl, 20.5; and PO_4, 5.3. The preservative-free Co^{60}-Vit. B_{12} (Merck) was adjusted to approximately 90 mμg/ml in 0.14 M potassium chloride. A Dubnoff water bath was used for incubations which were generally 3½ hours at 37°C under air atmosphere. The same Vit. B_{12} uptake was observed under air as under oxygen or nitrogen. Each 25 ml Erlenmeyer flask contained 1 ml of homogenate, 2 ml buffer, 0.2 ml Co^{60}-Vit. B_{12} solution, and 1.4 ml of isotonic potassium chloride which contained varying amounts of IFC.* After incubation the contents of each flask were transferred to a small test tube and centrifuged. The sediment was resuspended once in 4 ml of isotonic potassium chloride and again centrifuged. Supernatant was poured and drained from washed sediment and the radioactivity of Co^{60}-Vit. B_{12} bound in the sediment was determined in scintillation well.

Results. Fig. 1 shows rate of Vit. B_{12} uptake by rat liver homogenates and IFC with varying amounts of Vit. B_{12}. In this experiment flasks containing 5 times the regular quantities of materials were incubated at 37°, and 3 ml aliquots were withdrawn at intervals into iced tubes and centrifuged immediately. The IFC, WIF-54B from hog pylorus, had activity of 1.7 USP units/mg as shown by this homogenate-Vit. B_{12} uptake assay. When uptake in presence of IFC is corrected for control uptake by the homogenate, the curves in Fig. 2 are obtained. The action of IFC at 37° is so rapid that aliquots removed at zero-time showed considerable uptake: the process can be appreciably slowed by reducing the quantity of Vit. B_{12} and temperature. Despite this rapid action, for laboratory convenience all subsequent experiments were incubated for 3½ hours. Enhancement of IF activity by calcium as reported by Herbert (2) with rat liver slices was confirmed as shown in Fig. 3. Calcium was always present in the standard assay at final concentration of 8.2 mM/liter as indicated by arrow in the Figure.

Activities of IFC from hog pylorus were proportional to the initial uptake slope, mμg Vit. B_{12}/mg. IFC, as determined by a plot of Vit. B_{12} uptake *vs.* μg IFC. Typical plots are shown in Fig. 4. Depression of uptake after the peak may be caused by IF itself and not by an inhibitor (although see below), for when plots of IFC varying from 0.017-2.0 USP units/mg are adjusted to superimpose the ascending slopes, the descending slopes are often very similar. To correct for variation among rat livers, a standard IFC, 315-W9 having 0.2 unit/mg, was included in each set of assays, and all activities were calculated relative to

* The following abbreviations are used: IF, intrinsic factor; IFC, intrinsic factor concentrate. All IFC prepared from hog tissues.

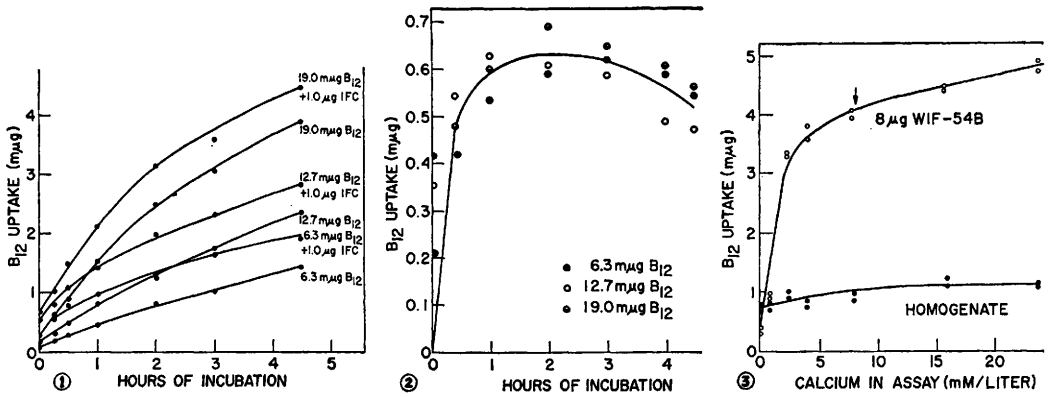
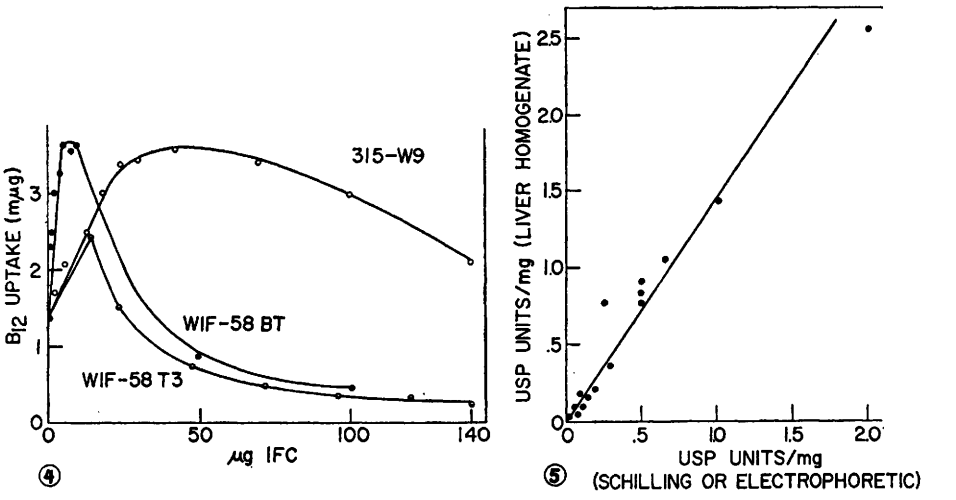


FIG. 1. Rate of vit. B_{12} uptake in presence of liver homogenate and IFC WIF-54B. Quantities are per 3 ml aliquots.

FIG. 2. Rate of vit. B_{12} uptake in presence of IFC WIF-54B (1.0 μg) after correction for uptake by homogenate.

FIG. 3. Uptake of vit. B_{12} by rat liver homogenate and WIF-54B (8 μg) as a function of Ca^{++} concentration. Ca^{++} concentration used in standard assays is shown by arrow.



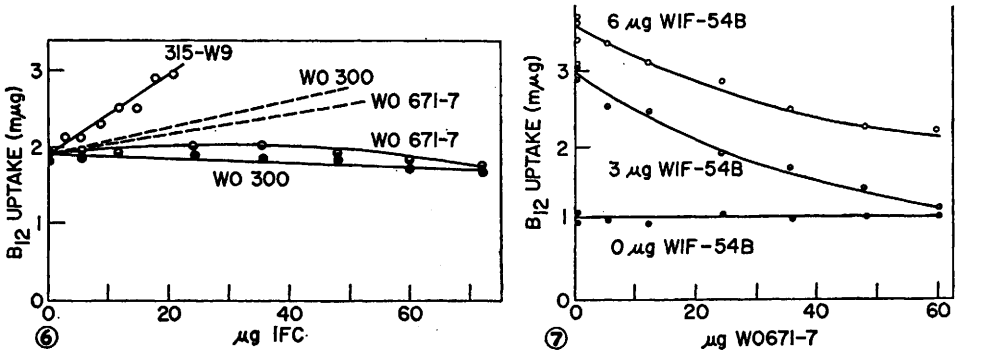


FIG. 4. Uptake of vit. B_{12} by varying amounts of 3 hog pyloric IFC.

FIG. 5. Comparative assays of 15 IFC from hog pylorus.

FIG. 6. Vit. B_{12} uptake by varying amounts of 2 hog duodenal IFC. Broken lines indicate uptakes expected from their Schilling potencies.

FIG. 7. Inhibition of WIF-54B, a hog pyloric IFC, by increasing quantities of WO671-7, a hog duodenal IFC.

it. Uptake by 315-W9 with 15 different liver homogenates was 98 ± 3 SEM mμg Vit. B_{12}/mg 315-W9. The uptake by these same homogenates without added IFC was less uniform. 2.1 ± 0.2 mμg Vit. B_{12}/ml hogomenate.

In Fig. 5 are plotted the potencies of many hog pyloric IFC as determined by homogenate uptake of Vit. B_{12} *vs.* their potencies by either the Schilling assay(6) or electrophoretic assay described by Barlow and Frederick(7). The correlation among the assays indicates that IF activity is being measured by the liver homogenate method. The line of least squares in Fig. 5 does not coincide with the expected 45° line for a 1:1 relationship between the assay methods. This is due, perhaps, to an error in ascribing a potency of 0.2 unit/mg to the reference standard 315-W9. The randomness of the points is due to combined variability of the 3 assay methods.

By use of this *in vitro* assay an inhibitor of Vit. B_{12} uptake in IFC from hog duodenum was detected as shown in Fig. 6. Preparations WO-300 and WO-671-7 had potencies of 0.067 and 0.050 USP unit/mg in the Schilling assay. The uptakes calculated from these potencies are indicated by broken lines in the Figure. As can be seen, these typical duodenal preparations cause a lower Vit. B_{12} uptake than would be expected from their potencies.

There is also evidence for an inhibitor in some IFC from hog pylorus. In these preparations the initial uptakes agreed with the Schilling assay but maximum uptakes were depressed. This effect is demonstrated in Fig. 4 by WIF-58T3. An inhibitory fraction could be isolated from such an IFC.† Inhibitory samples of duodenal IF also inhibited uptake of Vit. B_{12} by pyloric IF as shown in Fig. 7. When increasing amounts of the duodenal IFC were added to a fixed amount of pyloric IFC, inhibition is shown by the curves. The same duodenal inhibitor thus inhibited the action of IFC from both tissues. Whether the effect is on IF itself, Vit. B_{12}, or the homogenate was not established.

†O. Walasek, unpublished.

Further experiments with livers and gastric juices from other animal species revealed extreme specificity. This was also reported by Johnson and Driscoll(8). With rat liver homogenates, gastric juices from 4 other rats and 2 non-pernicious anemia human subjects either depressed or did not elevate the Vit. B_{12} uptake. IFC from hog pylorus did not increase Vit. B_{12} uptake by homogenates of livers from 8 mice, 2 hogs, 1 rabbit and 1 chick. This lack of interaction among various livers and IFs may be due to inhibitors as shown above. Herbert(3) has discussed a similar phenomenon.

It is evident that the liver homogenate assay, as described here, is a precise and convenient method for determination of hog pyloric intrinsic factor. The results obtained with this assay correlate very well with those of the Schilling technic in man.

Summary. A convenient laboratory assay for hog pyloric intrinsic factor using Co^{60}-Vit. B_{12} and rat liver homogenates is described. By means of this *in vitro* assay the presence of an inhibitory substance was detected in certain crude preparations derived from hog duodenum. These showed a diminished response relative to activity in the Schilling urinary excretion assay.

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