first hour, remaining constant thereafter for at least 5 hours. During this time the degradation of the hormone is intensified and no more than 15–18% of the recovered radioactivity represents immunologically active insulin.

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Studies on the Site of Synthesis of Transcobalamin-II* (32789)

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Mammalian serum binds exogenous radioactive vitamin B_{12} (B_{12}) and in most species, two or more protein binders have been separated by cellulose chromatography (1, 2). Two binders in human serum, characterized by Hall and Finkler have been designated transcobalamin-I (TC-I) and transcobalamin-II (TC-II). Mouse serum unlike the serum of other mammalian species appears to have only one B_{12} binder, characterized by Coffey *et al.* (3) which elutes from cellulose ion exchange columns at the same pH and which has the same molecular size, i.e., equivalent to proteins with molecular weights of 35,000 (4) as the TC-II of human serum. Tan *et al.* have demonstrated that TC-II of mouse, rat, dog, and human, all have a molecular size similar to proteins that have molecular weights of 35,000 (4). This size has been confirmed recently for human TC-II by Grasbeck (5). A rapid assay for TC-II has recently been achieved by Hansen *et al.* (6,7), employing zirconyl phosphate gel (Z-gel). Zirconyl phosphate gel of pH 6–7 specifically binds TC-II, but does not bind TC-I which if present, is present only in small amounts in normal human serum, but elevated in serum of chronic myelogenous leukemia patients (6). TC-I has a molecular size similar to proteins with molecular weights of 117,000 (5). The charcoal method devised by Miller (8) differentiates between protein bound and free B_{12}, and thus, the difference between the two assays represents TC-I (6). Zirconyl phosphate gel of pH 5 binds intrinsic factor of human, rat, mouse, and hog, R of normal human gastric juice, but does not combine TC-I (3,6,9,10). These B_{12} binders are not adsorbed

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by Z-gel of pH 6–7. Employing the zirconyl phosphate gel assay to determine TC-II, we did a number of studies to determine the site of synthesis of TC-II and these are reported below.

Vitamin $B_{12}{}^{57}Co$ was purchased from Philips-Duphar, Appololaan, 151, Amsterdam, Holland, and diluted with unlabeled vitamin B_{12}, (Squibb) to give solutions which had activities of 1500–1700 cpm/12-mμg or 15,000–17,000 cpm/mμg. Swiss mice were from a colony which had originally been started with mice purchased from A. R. Schmidt Co., Madison, Wisconsin, and rats from a colony originally started from Holtzman rats obtained from Madison, Wisconsin. Both, mice and rats were fed Purina Lab Chow diet (36 μg of vitamin B_{12}/kg of diet) *ad libitum*. Carbon tetrachloride was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey (reagent grade).

Assay of serum for transcobalamin-II. To assay mouse serum for transcobalamin-II, 0.25 ml of serum was pipetted into 20-ml disposable test tubes and mixed with 0.5 ml of solution containing 25 ng of $B_{12}{}^{57}Co$ (1700 cpm/mμg). Three ml of pH 7.0 ammonium acetate (0.1 *M* acetate) solution were added and followed with 5.0 ml of pH 7.0 Z-gel (11). The tubes were filled to 15 ml with the ammonium acetate solution (pH 7) and inverted. The solution was centrifuged at 3000 rpm for 5 min. The supernatant fluid was discarded and the gel sediments were washed twice with 12.0 ml of the ammonium acetate solution. The final supernatant fluid was discarded and the pellets were assayed for TC-II–$B_{12}{}^{60}Co$ complex with a well-type gamma scintillation counter. Transcobalamin-II concentration in mouse serum is greater than in that of the other mammalian species used.

Results. When vitamin B_{12} unsaturated TC-II of blood is saturated by intramuscular injection of cyanocobalamin, TC-II–B_{12} complex is formed, and this complex is taken up by tissues with subsequent destruction of TC-II; free vitamin B_{12} is found in the cells (11–13). If just enough cyanocobalamin is administered to an animal to saturate TC-II, it is in effect destroyed, the reappearance of TC-II in the serum represents newly synthesized TC-II, and thus, this method can be used to measure TC-II synthesis *in vivo*.

Both the liver and kidneys play key roles in vitamin B_{12} metabolism, and these were first studied as possible sites of TC-II synthesis. The role of the kidney in TC-II synthesis was studied by performing total nephrectomy of mice and measuring TC-II levels and rate of TC-II synthesis by these animals. Nephrectomized mice remained in excellent health for 24 hours after nephrectomy, and TC-II levels were identical to controls 24-hours post-nephrectomy. Mice which had been nephrectomized 16 hours previously were given enough cyanocobalamin by intramuscular injection to give total saturation of circulating TC-II. As may be observed, synthesis of TC-II by nephrectomized animals was identical to that observed in sham operated control animals (Fig. 1).

A preliminary experiment was then carried out to determine whether the liver was the site of TC-II synthesis. The known hepatotoxic chemical, carbon tetrachloride was employed. To determine the effect of carbon tetrachloride on TC-II synthesis, 0.5 ml was mixed with 9 volumes of mineral oil, and 2 ml of the mixture injected intraperitoneally into groups of mice. Animals were sacrificed at 12, 24, and 48 hours. Control mice received 1.53 ml of mineral oil intraperitoneally. Frozen sections of liver were obtained, fixed with 10% formaldehyde, and stained with hematoxylin–eosin. The histological examination revealed approximately one third of the liver had been destroyed and this correlated well with the effect of carbon tetrachloride on serum TC-II levels. After 24 hours, the serum TC-II levels of experimental animals which received carbon tetrachloride were approximately two thirds of that of control animals which received only mineral oil. Carbon tetrachloride was then administered intraperitoneally and just enough cyanocobalamin was injected intramuscularly to saturate TC-II. Control animals received mineral oil and cyanocobalamin. The rate of reappearance of TC-II in experimental and control mice is shown in Fig. 2. The rate of synthesis of TC-II is markedly decreased and TC-II levels in the treated mice did not return to normal in 18 hours. Again, the liver

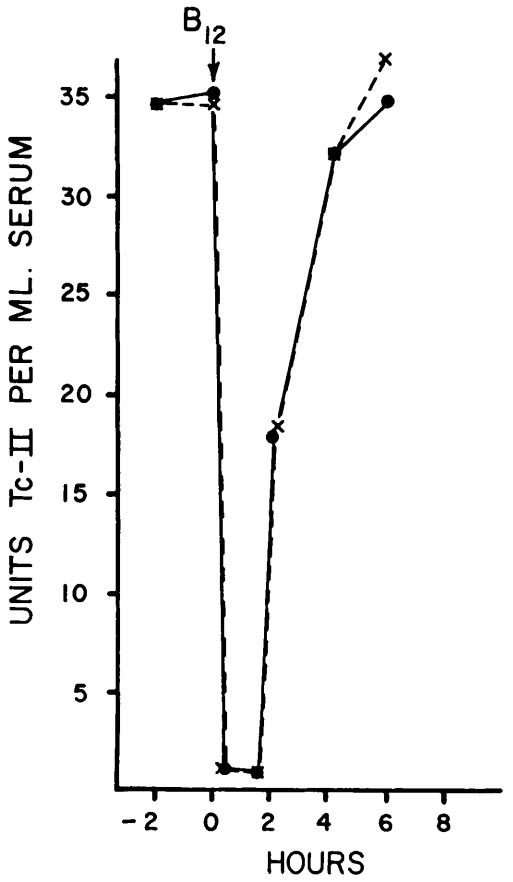

FIG. 1. Effect of total nephrectomy on reappearance of serum TC-II after saturation of TC-II with vitamin B_{12}. Each value represents the TC-II concentration of pooled sera of 10 mice, 1 μg of B_{12} given by intramuscular injection; (X) nephrectomized mice; (●) sham operated control mice.

damage as evaluated histologically correlates well with the effect of carbon tetrachloride on TC-II synthesis, and this experiment implies that in the mouse, the liver is the site of TC-II synthesis.

To further demonstrate that TC-II is synthesized by liver, a liver perfusion experiment was carried out. The perfusion apparatus and procedure were similar to that described by Schmimassek (14). Results of two perfusion experiments are shown in Fig. 3. A steady increase of unsaturated TC-II in the perfusion medium was observed. In these experiments, the TC-II levels of the perfusion medium increased from 0.2 units to 0.9 and 0.7 units of TC-II per ml of cell free perfusate. The total cell free perfusate was 125 ml, thus, this increase represented a total output of 87.5 units of TC-II in one experiment, and 62.5 units of TC-II in the second experiment.

The possibility that transcobalamin-II represents modified intrinsic factor or is synthesized by the small intestine, has been considered in a recent article by Grasbeck (5). Table I demonstrates TC-II levels of serum obtained from normal persons, pernicious anemia patients, patients with radical small bowel resections, and patients with total gastrectomy. Transcobalamin-II serum levels were found to be normal or higher in the pernicious anemia patients and gastrectomy patients than in the normal controls. We have established that the B_{12} binder measured by the Z-gel assay is Transcobalamin-II by employing DEAE cellulose ion exchange column chromatography, and chromatography of serum on G-100 Sephadex columns.

Table II shows the TC-II levels of a mongrel dog which underwent radical gastrectomy. In this animal, the lower esophagus was mobilized and sutured to the duodenal stump in an end-

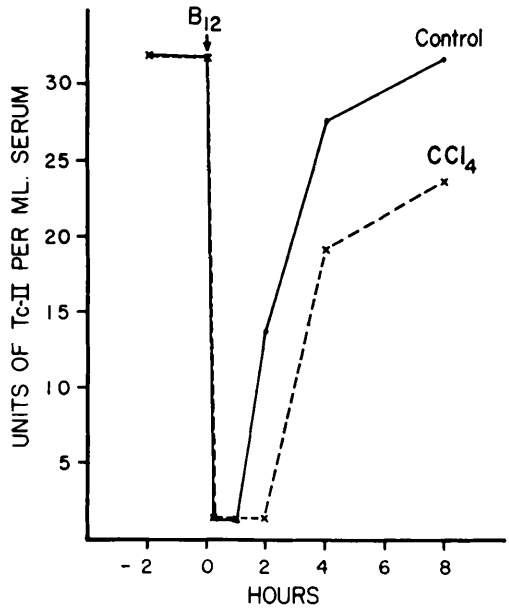

FIG. 2. Effect of carbon tetrachloride on the reappearance of TC-II in serum after saturation of TC-II with vitamin B_{12}. Each value represents TC-II concentration of pooled serum of 10 mice; 1 μg of B_{12} given by intramuscular injection.

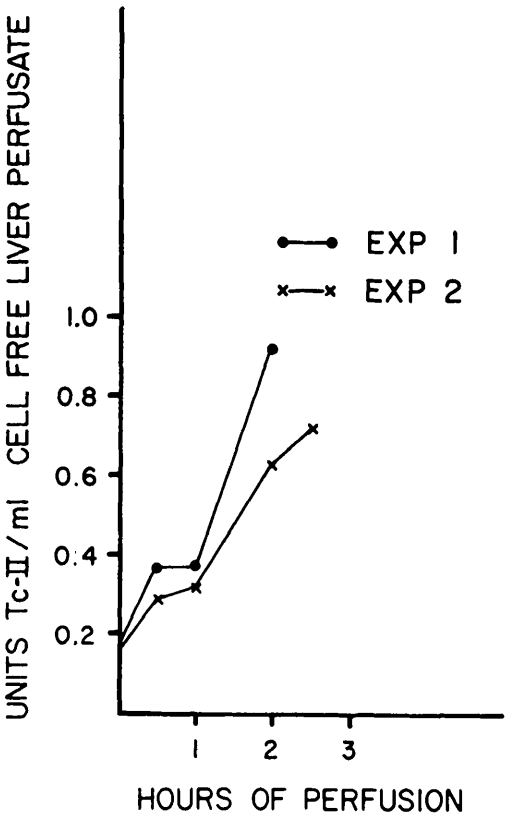

FIG. 3. Synthesis of transcobalamin-II by perfused liver. See text for details.

to-end anastomosis. During the experimental period, a steady weight loss was observed with a decline of serum protein primarily in the albumin fraction. In spite of a decline of serum protein albumin, there was no significant decrease of serum TC-II. At the end of 4 months, cyanocobalamin (10 μg) was administered to saturate the TC-II. The reappearance of TC-II in the serum is shown in Fig. 4, and is identical to results obtained with normal dogs.

Discussion. The data presented apparently indicate that neither the stomach nor the kidney play a role in the synthesis or destruction of transcobalamin-II. It was necessary to rule out the stomach as the site of TC-II synthesis because of the possibility that TC-II was modified intrinsic factor which in humans and rats has a molecular size equal to proteins with molecular weights of 50,000–60,000 (15–18). The marked difference in chemical properties of transcobalamin-II and intrinsic factor could have been explained by the degradation of intrinsic factor to TC-II, which has a molecular size equal to proteins with molecular weights of 35,000.

The above observations that TC-II levels of patients with pernicious anemia, gastrectomy, as well as the observation that the gastrectomized dog maintained a normal TC-II level for several months, demonstrate that TC-II of man and dog is not derived from intrinsic factor, and it seems valid to assume that TC-II of dog and man is synthesized by liver. A similar conclusion has been reached recently by Grasbeck *et al.* (5). The experiments presented above demonstrate that the liver is probably the primary site of TC-II synthesis. A total of 87.5 units of TC-II was synthesized in one liver perfusion during a 2-hour experiment, and 62.5 units of TC-II synthesized in the second liver perfusion in 2.5 hours. The normal concentration of TC-II in rat serum as determined by Z-gel assay is 3–4 units/ml. In human and animal studies, we have found TC-II to equilibrate between the vascular system and the intestinal fluid and to have a 2–4-hour half-life (11–13). Thus,

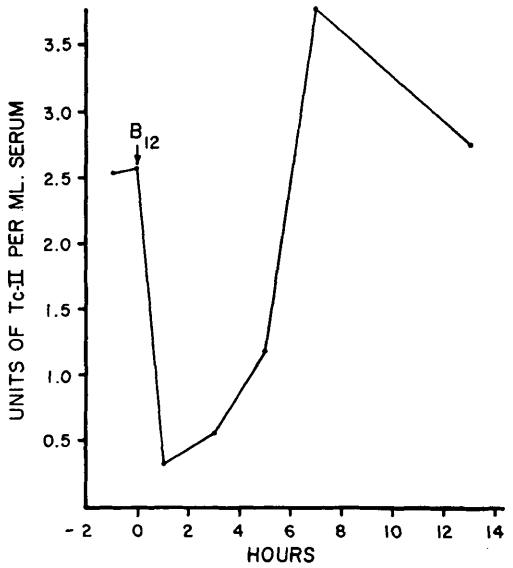

FIG. 4. Reappearance of TC-II in serum of a gastrectomized dog after saturation of TC-II with B_{12} (4 months post operative). A 10 μg dose of B_{12} was given iv to saturate TC-II. See text for details.

TABLE I. Assay of Serum Transcobalamin-II in Normal Persons, Pernicious Anemia Patients, Gastrectomized Subjects,[a] and Patients with Small Bowel Resections.

	Units of TC-II per ml (pH 7.0 Z-gel)
Normal (10)[b]	0.96 (0.86–1.06)[c]
Pernicious anemia (10)	1.39 (0.91–1.80)
Gastrectomy (3)	0.80 (0.76–0.90)
Small bowel resection (2)	1.2 (1.0 –1.3)

[a] Data were collected in a period of 6 months. Further studies are now in progress.

[b] Figures in parentheses represent the number of patients tested.

[c] Figures in parentheses indicate the ranges of results, and those without represent the mean values.

TABLE II. Serum TC-II of a Gastrectomized Dog.

Time (months)	Units of TC-II per ml	mg of albumin/ml
Preoperative	2.6	35
Postoperative 1	3.0	30
2	2.0	25
3	2.5	25

the amount of TC-II synthesized by the liver in 2 hours is in agreement with *in vivo* results.

Although the small intestine has not been ruled out in these studies as a source of TC-II synthesis, the demonstration of normal TC-II levels in serum of patients with radical small bowel resections cast doubt that the intestine is the source of TC-II in man.

Summary. The liver, kidneys, and stomach were studied to determine the site of transcobalamin-II synthesis. The kidney is not the site since total nephrectomy had no effect on the reappearance of TC-II after saturation of TC-II with B_{12}. Total gastrectomy also had little effect on TC-II levels or rate of synthesis. The liver proved to be the site of TC-II synthesis because of the following findings. Destruction of liver with carbon tetrachloride resulted in a decrease of serum TC-II. Unsaturated TC-II in the blood after saturation with cyanocobalamin was markedly low in the carbon tetrachloride treated animals. TC-II was shown to be synthesized by the perfused rat liver.

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