Material and Methods

Serum

Cord blood was collected from the placental parts of umbilical cords immediately after normal deliveries at the Albany Medical Center Hospital, Albany, N.Y. Ten-fifteen ml of blood was taken from one placenta. The serum was separated by gentle centrifugation within four hours. It was either refrigerated for not more than 20 hours prior to use, or frozen immediately after separation and stored at $-20^\circ\text{C}$ until used. No visibly hemolyzed samples were used. To check the possible effects of hemolysis, some grossly hemolytic serum samples were studied by DEAE-chromatography and no differences attributable to hemolysis were found.

The samples were collected from 35 normal deliveries, occurring at daytime between October 25, 1966 and June 14, 1967. Sixteen of the babies were boys and 19 girls. Their birth weights ranged from 2530 to 4680 grams, the average birth weight in the series being 3370 grams.

Large scale preparative studies were performed on pooled serum from the above mentioned cord serum samples and on cord serum obtained from Grand Island Biological Corporation, Grand Island, N.Y. (catalog number 619). This specially ordered serum was prepared in the cold, immediately frozen, shipped in the frozen stage and stored at $-20^\circ\text{C}$ until used. The serum was delivered in 20 ml and 50 ml lots.

Infant blood was collected at the Flower and Fifth Avenue Hospital, New York, N.Y. Three serial samples were obtained from three healthy infants at different time intervals between 1 day and 30 days of age. Individual samples were collected from five healthy infants and from eleven infants hospitalized at the Flower and Fifth Avenue Hospital for various non-hematological disorders. Their ages were between 1 day and 158 days at the time of blood collection. The sera were separated and processed as described above.

Maternal blood was obtained from the mothers of seven of the above mentioned healthy infants. The maternal blood was drawn the day after the delivery. Additionally, blood was obtained from five healthy women in active labor at the Albany Medical Center Hospital, right before delivery. The maternal samples were processed and stored identically with the infant and cord blood samples.

Normal adult blood was obtained from subjectively healthy male and healthy, non-pregnant female laboratory personnel. Serum was separated and stored as above.

Vitamin B$_{12}$

The radioactive vitamin B$_{12}$ used in this work was $^{57}$Co-labeled cyanocobalamin (B.P.). It was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England, through Merek and Co Inc., Rahway, N.J. Three batches with the code CT.2P were used:

- Batch 37, specific activity 45 µCi/µg on July 20, 1966. According to the manufacturer, the radiochemical purity of the batch was 95% by dilution and electrophoretic analyses.
- Batch 48, specific activity 70 µCi/µg on January 24, 1967. Radiochemical purity 98%.
- Batch 61, specific activity 110 µCi/µg on July 29, 1967. Radiochemical purity 98%.

Aqueous dilutions, prepared from the above batches, gave working solutions with suitable concentrations of vitamin B$_{12}$. These working solutions were kept in the dark at $+4^\circ\text{C}$ and not used for more than one month after the dilution.

The non-radioactive vitamin B$_{12}$ used in this work was USP reference standard nonradioactive cyanocobalamin.

The measurement of radioactivity

The measurement of radioactivity was done by scintillation spectrometry. A well-type crystal detector connected to a scintillation spectrometer (Baird Atomic 530) with an automatic sample changer was used. The radioactivity of the samples was calculated using $^{57}$Co standards of corresponding volumes. Counts per minute (cpm) for the standards were recorded daily and their actual radioactivity calculated using a half-life of 273 days for $^{57}$Co. The counting efficiency of the system was 1.8 × 10$^4$ cpm/µg. The background varied between 30 and 40 cpm. A counting
time to provide a minimum of 1000 counts was always used for the radioactive samples, although the amount of counts recorded was usually higher.

The labeling of the B₁₂ binders

All the superscripted B₁₂ labeling of serum proteins was done in vitro. If not otherwise mentioned, the amount of radioactive vitamin B₁₂ added was 300 pg per 1 ml of serum, an amount which is well within the normal unsaturated B₁₂-binding capacity of serum proteins (e.g. Miller 1958). The working solutions of superscripted B₁₂ contained 300 pg in 0.14—0.21 ml.

The radioactive vitamin was added dropwise into the serum with gentle shaking. After incubating in a +37°C water-bath for 20 minutes, the serum sample was dialyzed against the starting buffer of the next procedure, to remove any unbound B₁₂ and to equilibrate the sample. The serum sample was counted for radioactivity prior to and after the dialysis. Also the dialysing fluid was counted and no radioactivity could be detected in it when the standard amount of superscripted B₁₂ was added.

The labeled serum was then submitted for various procedures in order to fractionate the vitamin B₁₂ binders and to characterize them.

DEAE-cellulose chromatography

Diethylaminoethyl (DEAE) ionic exchange cellulose type 40 was purchased from Carl Schleicher & Schuell Co, Keene, N.H. The capacity of the cellulose was 0.92 meq/g. The new cellulose powder was suspended in water, stirred and allowed to sedimentate. The yellowish supernatant containing the finest particles was decanted off. This was repeated twice. The cellulose was then suspended in 0.5 N NaOH and stirred for 30—30 minutes. The alkali was washed off with water until the pH of the washing water reached 7.0. The same treatment was repeated with 0.5 N HCl and then again with 0.5 N NaOH. When the last alkali had been washed off, the cellulose was suspended in 0.01 M phosphate buffer, pH 8.0 and the pH of the mixture brought to 8.0 with 0.3 M NaH₂PO₄. The buffer was filtered off, the cellulose washed five times with 0.01 M pH 8 phosphate buffer and suspended in it. The pH was again checked and readjusted, if necessary. Two three drops of toluene per 1 liter of the mixture was added as a preservative. The cellulose was stored in the cold. Only cellulose processed from new stocks was used. DEAE-Sephadex A-50, capacity 3.9 meq/g, made by Pharmacia, Uppsala, Sweden, was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. It was processed as above using 0.2 N acid and alkali, and suspended in 0.01 M phosphate buffer pH 8.0.

The methods used for DEAE-cellulose chromatography were essentially the same as those used and described by Hall and Finkler (1965). Two modifications of the chromatography technique were used. The so-called "mini-column technique" was used to study the B₁₂-binding pattern of small serum samples, in the range of 1 ml. Columns with an internal diameter of 1 cm were packed with the DEAE-cellulose slurry in 0.01 M phosphate buffer, pH 8.0. The packing was performed in 3—4 steps, first by gravity and then by applying an air pressure of ten pounds on the column. The final length of the column was 3 cm. The labeled, dialyzed serum sample was layered on the column and washed in with the starting phosphate buffer. A concave phosphate buffer gradient of increasing molarity and decreasing pH, as modified by Hall and Finkler (1965) after Peterson and Chinazze (1962), was used to elute the column. To produce the gradient, a nine-chambered, continuous-flow device was used. The starting buffer was 0.01 M pH 8.0 and the limiting buffer 0.3 M pH 4.5. The volume of each chamber was 25 ml. The flow rate in different columns varied between 2.5—7.0 ml/hr without any effect on the separation. Fractions of 5 ml were collected and assayed for protein and radioactivity content. The fractions from these small columns were not used for further separation or characterization of the B₁₂ binders.

The so-called "big column technique" of DEAE-chromatography was used to process large amounts of labeled serum for further processing by other methods. DEAE-cellulose was packed to the height of 20 cm in a column with an internal diameter of 4 cm. The packing was performed in 4—5 steps, first by gravity and then by 10 pound air pressure. Up to 50 ml labeled serum was processed with these columns. The elution gradient was the same as described for "mini-columns", the volume of each chamber being 300 ml. The experiments were performed at a temperature of +4°C with a flow rate of 30—40 ml/hr. Fractions of 30—40 ml were collected and aliquots taken for protein, radioactivity, and B₁₂ assay. The fractions were pooled according to the radioactivity distribution, concentrated by ultrafiltration and dialyzed for subsequent procedures.

CM-cellulose chromatography

Carboxymethyl (CM) ionic exchange cellulose (Whatman column chromatia CM 11, made in England by W & R Balston Ltd., nominal capacity 0.6 meq/g) was washed successively with ten volumes of 0.5 N HCl and 0.5 N NaOH. The acid and alkali were
washed off with water, the washed cellulose was suspended in 0.02 M sodium chloride-sodium acetate buffer pH 5.4, and the pH of the mixture adjusted to 5.4. Thereafter the cellulose was rinsed several times with the above mentioned acetate buffer, resuspended in it and stored at +4°C. No used CM-cellulose was employed.

A slurry of the cellulose in 0.02 M acetate buffer pH 5.4 was poured into a column with an internal diameter of 1.5 cm and allowed to settle by gravity to the height of 45 cm. The sample, which had been dialyzed against the same acetate buffer, was layered on the column and washed in. The first elution was performed with 0.02 M acetate buffer pH 5.4, followed by 0.027 M phosphate buffer pH 7.0, and completed with 0.2 M phosphate buffer pH 7.8. The outlet of the column was connected to a UV-scanner which indicated the elution of the protein fractions. The buffer was changed after the elution of a protein peak.

The flow rate was kept at 60—80 ml/hr and fractions of 14 ml were collected and counted for radioactivity. The fractions were pooled according to the radioactivity peaks and concentrated for further use.

**Gel filtration on Sephadex®**

*Sephadex G-200*, made by Pharmacia, Uppsala, Sweden, was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. The buffer used in the gel filtration experiments was 0.05 M phosphate buffer pH 7.4, made 0.5 M for NaCl and containing 0.02 % sodium azide as preservative.

The gel was allowed to swell in the buffer for 72 hours prior to use. A slurry of it was poured to the height of 70 cm in a silicone coated column with an internal diameter of 2.5 cm. The column was stabilized with the buffer for 24 hours. The same column was used repeatedly.

The dialedyzed sample (volume 1—2 ml) was gently layered on the column, allowed to enter and washed in with three times 1 ml of the gel buffer. The column was eluted with the gel buffer at a flow rate of 6—12 ml/hr. The outlet of the column was run through a photocell recording the absorption at 280 nm. Fractions of 2 ml were collected and counted for radioactivity. Either the UV-recorder or a chemical assay was employed to determine the protein concentration.

The molecular weight estimations from the gel filtration experiments were based on the linear correlation between the elution volume of a molecule and the logarithm of its molecular weight (Andrews 1964). The amount of buffer needed to elute the peak fraction was taken as the elution volume of the protein. The void volume for the column was measured equally with Blue Dextran 2000 (Pharmacia). In order to eliminate the effect of possible alterations in the column during prolonged use, the ratio of elution volume to void volume was used instead of elution volume alone.

The following set of reference proteins (from Mann Research Laboratories Inc., New York, N.Y.) was used to produce the standard line illustrated in figure 1:

- Chymotrypsinogen, molecular weight 25,000
- Ovalbumin, molecular weight 45,000
- Bovine albumin, molecular weight 67,000
- Human gamma-globulin, molecular weight 160,000

This standard line was used to estimate a molecular weight value for the filtrated samples. Sephadex G-100 was treated similarly as G-200 and a column with the dimensions of 2.4 X 85 cm was prepared. This column was used to fractionate 1—2 ml samples of labeled serum. The protein distribution pattern recorded by the absorption at 280 nm was used as a reference to the location of the radioactivity peaks. No molecular weight estimations were performed on this column.
Electrophoresis

Geon block electrophoresis was performed using essentially the same methods as Hall and Finkler (1962). Geon number 427 polyvinylchloride resin was purchased from B. F. Goodrich Co., Niagara Falls, N.Y. The buffers used were 0.06 M barbiturate buffer pH 8.6 and 0.1 M sodium acetate-sodium chloride buffer pH 4.5.

The resin was suspended in water, stirred and filtered. The same treatment was repeated twice using the buffer. A wet, semi-firm slurry was made with the buffer and poured into three, separated, parallel blocks of 45 cm X 8 cm X 2 cm in the electrophoretic apparatus. The gel was connected to the buffer compartments with well soaked gauze bridges. The samples (CM-purified binders or in some instances labeled serum) were dialyzed against the electrophoresis buffer and applied at the center of the block in a transverse slit of about 2-3 mm. The volume of the sample varied between 0.2-0.5 ml, depending on the level of concentration achieved by the preceding ultrafiltration and judged by the radioactivity of the sample. The starting voltage was 200 V (= 4.4 V/cm) and the running time 18-20 hours. The block was kept at a temperature of +4°C by a built-in cooling system in the electrophoretic apparatus. After the run the block was dried for 1-1.5 hours and then cut into 16 transverse segments of 1 cm. Each segment was counted for radioactivity and the total cpm per segment recorded. The distance from the point of application for the segment containing the highest amount of cpm was taken as the mobility of the fraction. The electrophoretically separated fractions were not used for further studies. The distribution of serum proteins recorded in a few experiments was measured by eluting the segments with 0.01 M phosphate buffer pH 8.0 and assaying the eluates for protein.

Uptake of $^{57}$CoB$_{12}$ by HeLa cells

The technique used was essentially the same as that described by Finkler and Hall (1967). HeLa cells in monolayer cultures were generously provided by Dr. J. V. Landau, Ph.D., the Biology Section of Veterans Administration Hospital, Albany, N.Y. The cell growth medium was decanted off and the dialyzed sample (CM-purified binders in a volume of 1-2 ml) with Hank’s base 199-IX (from Grand Island Biological Corporation, Grand Island, N.Y., catalog number 115 H) was added to the cells in a total volume of 12 ml. The cell bottles were then placed at +37°C for 2 hrs. After the incubation the medium was decanted off and the cell layers washed twice with buffered, ice-cold solution containing 0.8 % NaCl, 0.04 % KCl, 0.1 % dextrose, and 0.035 % NaHCO$_3$. The cells were then scraped from the bottle with a rubber-tipped spatula and washed three times with centrifugation in the ice-cold buffered saline solution. The washed cells were suspended in 5 ml of the same solution. The amount of cells was counted in a hemocytometer using the standard laboratory technique for leukocyte counting. The cell suspension was counted for radioactivity.

Assay of vitamin B$_{12}$

The assays of vitamin B$_{12}$ were kindly performed by Mr. Edward S. Allen, using the Euglena gracilis z-strain and the method described in detail by Hall and Allen (1964).

Concentration of the samples

The fractions or pooled fractions from the chromatographic procedures were concentrated for further use by ultrafiltrating them through Visking dialysis tubing (obtained from Union Carbide Corporation, Chicago, Ill.). The tubing was thoroughly washed, filled with the sample, and placed into reduced pressure in a vacuum flask at +4°C. The inside of the tubing was kept in open connection with the outside air. The reduced pressure was adjusted to a level which gave maximal expansion of the tubing without breaking it. Possible leaks were detected by counting the ultrafiltered outside fluid for radioactivity.

Dialysis

The dialysis of the samples against the appropriate buffer was performed in bags of Visking dialysis tubing. For DEAE-chromatography on "mini-columns" a short dialysis of 1-2 hours against 250 ml of the buffer was used. For other purposes the samples were dialyzed three successive times against 30-40 fold volumes and for 16-24 hours. Dialysis was performed at +4°C and a magnetic stirrer was used to mix the fluid.

Protein determination

The protein concentration in the chromatography fractions was measured by the method of Lowry et al. (1951). The protein distribution was used as a reference to the location of the radioactivity peaks. Hence, only the optical densities from the photometer were recorded, and the actual protein concentrations were not calculated.

All the reagents used in the present work were of "Analar Grade" quality.