body on this tumor appears to be qualitatively similar to that reported for other transplantable tumors, and may account for the rejection of Sarcoma I homografts.

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Separation of Bound and Free Vit. B_{12} on Sephadex G-25 Column.* (27764)

MAMORU KAKEI AND GEORGE B. JERZY GLASS
Gastroenterology Research Laboratory and Section of Gastroenterology, Department of Medicine, New York Medical College, Flower and Fifth Ave. Hospitals, New York

In the study of Vit. B_{12} metabolism and intrinsic factor the determination of free and bound Vit. B_{12} is of primary importance. Several methods have been developed for separating free from bound Vit. B_{12}, among the most popular of which are those of dialysis (1,2) and ultrafiltration(3) with the use of radioactive Vit. B_{12}. There are some disadvantages, however, to the use of these technics, such as dissociation of bound Vit. B_{12} (4) and partial denaturation of the binder during the procedure, as well as variability of results, depending on the variant of the technic used.

Gel filtration on Sephadex columns has recently been introduced(5-7) to achieve a 'molecular sieving' of materials of different molecular size. This has been used in our laboratory for fractionation of large molecular weight materials in gastric juice for the last 2 years(8). The molecular weight of Vit. B_{12} is about 1,300, and the molecular weight of Vit. B_{12} binders has consistently been found to be above 5,000(9). It occurred to us therefore that Sephadex G-25, which excludes materials of molecular weight over 3,500-4,500, would be well suited for separation of free from bound Vit. B_{12}. While this work was in progress, Daisley reported the applicability of Sephadex to the study of Vit. B_{12} in ocean water(10). A preliminary report of our work has been presented and published in abstract form(11).

Materials and methods. Sephadex G-25 columns were prepared according to instructions of Porath and Flodin(5-7). 6-20 g of dry Sephadex was suspended in borate buffer, pH 9.0, $\Gamma/2$ ranging from 0.06 to 0.24, and poured into the columns. The column sizes ranged from 0.8-1.7 by 32.0-50.0 cm and their total bed volumes were from 32.0-114.0 ml. The "void volumes" (Vo)(7) were from 11.0-43.0 ml and the 'inner volumes' (Vi) from 13.0-46 ml. Various amounts of dialyzed and lyophilized normal human gastric juice, ranging from 10 mg dissolved in 1.0 ml of buffer, to 100 mg dissolved in 14.0 ml of buffer, were applied on top of the column and eluted with the same buffer in which it was dissolved. The rate of elution was from 3.0-10.0 ml per hour, and the size of fractions collected was from 1.0-3.0 ml, depending on

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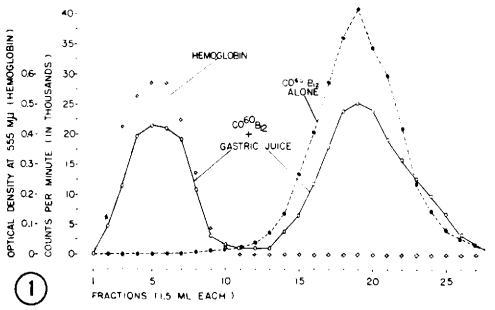
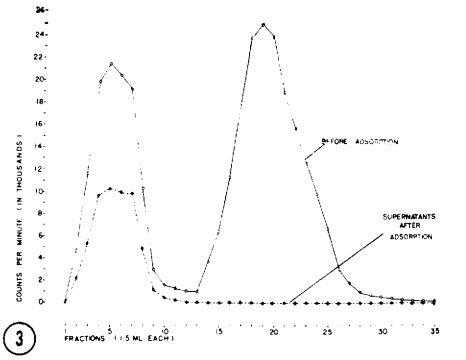
the size of the columns. The optical density of eluted fractions was read on the Beckman-DU spectrometer at 280 mμ.

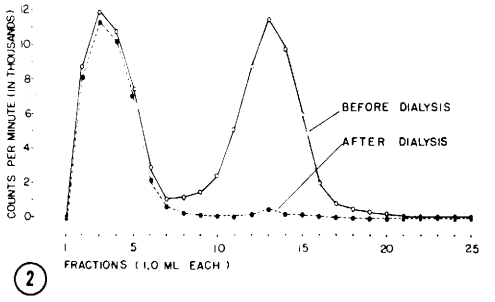
The radioactive Vit. B_{12} used in these studies was either $Co^{60}B_{12}$ of specific activity 0.86-0.97 μc/μg, or $Co^{57}B_{12}$ of specific activity 4.1 μc/μg. The labeled Vit. B_{12} was added to dialyzed and lyophilized gastric juice at levels of from 50-113 mμg/mg; amount of radioactivity on columns was from 1.0-20.7 μc. The fractions collected were counted in the heavily shielded counter provided with NaI-Thallium well shaped crystal, attached to gamma-analyzer and scaler. The counts were determined at the optimal setting for the energy spectrum of the particular isotope used. This reduced the background to 20 cpm and yielded a high statistical significance of the counts, with the counting efficiency of 66% for Co^{57}. Each material before counting was brought to the same volume of 4 ml to provide a similar geometry of counting.

Paper electrophoresis of the effluents from the column was performed by the method described previously from our laboratory with the use of Spinco vertical electrophoretic cells, borate buffer (Γ/2 0.06-0.24), Whatman No. 1 paper, at 120 v., 0.4 mA/cm, for 5½ hours(12,13).

Results. Elution of Free $Co^{57}B_{12}$. Free Vit. B_{12}, as expected, penetrated the gel of the Sephadex G-25 column and was eluted in the effluent in Peak II only (Fig. 1). This was in contrast to the elution pattern of the large molecular weight materials, such as hemoglobin, which when eluted from this column was excluded from Sephadex gel and appeared immediately after the Vo in Peak I (Fig. 1). *Elution of a mixture of $Co^{60}B_{12}$ with a normal, dialyzed and lyophilized gastric juice.* 4.8 μg $Co^{60}B_{12}$, containing 4.0 μc of Co^{60}, were added to 60 mg of dialyzed and lyophilized normal human gastric juice. This was far in excess of the Vit. B_{12} binding capacity of this material. The mixture was placed on a Sephadex column and eluted with a borate buffer (pH 9.0, Γ/2 0.06) at a rate of 6 ml/hour. One and a half ml fractions were collected and counted for radioactivity. Radioactivity was distributed within 2 peaks, Peak I, appearing immediately after Vo, and Peak II, appearing at the end of elution (Fig. 1). In another similar fractionation (not shown in Fig. 1) optical density read at 280 mμ was associated with the radioactivity of the material eluted into Peak I. This indicated that Vit. B_{12} eluted in Peak I was associated with protein or peptides derived from gastric juice. There was no exact correlation however between radioactivity and optical density curves. This is obviously due to the fact that only some proteins and peptides of gastric juice, which have been eluted into Peak I have Vit. B_{12} binding ability. This has been demonstrated previously(14,15). *Dialysis of materials eluted from the column.* In another experiment, 1.13 μg $Co^{60}B_{12}$ with 10 mg normal human lyophilized gastric juice were dissolved in borate buffer of pH 9.0 and the mixture was eluted from a Sephadex G-25 column with same buffer at a flow rate of 3.0 ml/hour. Each of the 1.0-ml fractions was counted for radioactivity, then dialyzed for 14 hours in small Visking tubes against running cold tap water. The dialysates were counted again for radioactivity. The results are shown in Fig. 2. As before, the radioactivity of the effluent, before dialysis, spread into 2 distinct peaks. All the radioactivity of Peak I of the effluent remained unchanged after dialysis. By contrast, the radioactivity of all fractions forming the second peak of the effluent was practically reduced to zero after dialysis. This shows that the radioactive Vit. B_{12}, which was eluted in Peak II of the column, was in a dialyzable, free form, while that contained in Peak I was undialyzable and represented bound Vit. B_{12}. *Charcoal adsorption of materials eluted from the column.* 60 mg of dialyzed and lyophilized pooled gastric juice from normal humans was dissolved in 12 ml of borate buffer of pH 9.0, containing 4.8 μg of $Co^{60}B_{12}$ having a specific activity of 0.86 μc/μg. The materials were eluted with the same buffer, and 35 fractions of 1.5-ml volume each were collected. Each of these fractions was counted for radioactivity, then mixed with 0.1 g Norit A, stirred for 15 minutes, and after high speed centrifugation at 25,000 $\times$ g for 15 minutes the supernatants were decanted and counted for radioactivity.







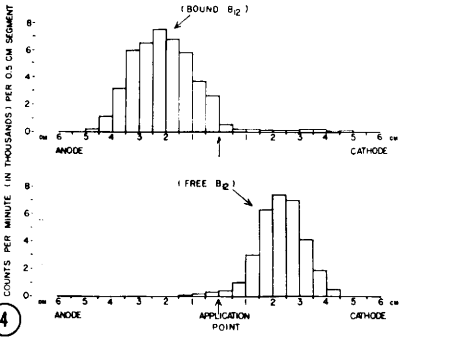


FIG. 1. Elution pattern on Sephadex G-25 column of 1) hemoglobin (40 mg in 8 ml borate buffer; 2) $Co^{60}B_{12}$ (3.2 μg in 8 ml borate buffer); 3) $Co^{60}B_{12}$ (4.8 μg) plus normal lyophilized human gastric juice (60 mg in 12 ml borate buffer). Void volume (Vo = 21 ml) preceding the first peak is not shown. Column dimensions, 1.6 × 32 cm.

FIG. 2. Elution pattern on Sephadex G-25 column of a mixture of $Co^{60}B_{12}$ (1.13 μg) with lyophilized normal human gastric juice (10 mg in 1 ml borate buffer), followed by extensive dialysis of the eluted fractions. Vo = 11.5 ml preceding the first peak is not shown. Column dimensions, 0.8 × 50 cm.

FIG. 3. Elution pattern on Sephadex G-25 column of a mixture of $Co^{60}B_{12}$ (4.8 μg) with normal lyophilized human gastric juice (60 mg in 12 ml borate buffer) followed by adsorption of the eluted fractions with Norit A (100 mg). Void volume is not shown in elution diagram. Same column as in Fig. 1.

FIG. 4. Electrophoretic patterns of radioactivity of the 2 fractions eluted from the Sephadex G-25 column after separation of a mixture of $Co^{57}B_{12}$ (5 μg) and normal lyophilized human gastric juice (100 mg in 14 ml borate buffer). Vo = 38 ml. Column dimensions, 1.7 × 48 cm.

The results obtained are shown in Fig. 3. Before charcoal adsorption the radioactivity was spread again into 2 peaks. After adsorption with charcoal, which is known to adsorb free Vit. B_{12}(16), radioactivity of the second peak was reduced to zero. This confirms previously reached conclusions that radioactive Vit. B_{12} contained in the effluent was in a free form, which resulted in its adsorption by activated charcoal. However, shaking of fractions from Peak I with Norit A reduced their radioactivity considerably. From the Norit adsorbed material 56% of the original radioactivity and 59% of the original optical density could be recovered by extraction with 4.5 ml 1 n. NaOH. More than one-half of bound Vit. B_{12} was therefore absorbed by Norit A. Thus Norit A adsorbs all free Vit. B_{12} and some of the Vit. B_{12} binding (peptide) materials from gastric juice as well.

Paper electrophoresis of materials eluted from the column. 5 μg of $Co^{57}B_{12}$ of high specific activity, together with 100 mg of lyophilized and dialyzed human gastric juice, were dis-

solved in borate buffer and eluted with the same buffer on Sephadex G-25 columns. Materials collected in Peak I and Peak II of the effluent were pooled separately, then dialyzed and lyophilized. Each of the 2 lyophilized fractions was then submitted to paper electrophoresis, as described above. Paper electrophoretic strips were cut into segments 0.5-cm wide and counted for radioactivity under similar geometry. The results are seen in Fig. 4. All the radioactivity in Peak I had anodic mobility. Its localization on the electrophoretic partition corresponded to that which was shown in our previous work(14,15) to be characteristic for intrinsic factor-related Vit. B_{12} binders in human gastric juice. On the contrary, radioactivity present in the second peak of the effluent had an exclusively cathodic mobility, and its localization on electrophoretic partition was typical of free Vit. B_{12}(15).

Discussion. Fractionation on Sephadex G-25 columns achieves a good separation of free and bound Vit. B_{12} contained in normal human gastric juice. Vit. B_{12} eluted into the second peak of the effluent from Sephadex G-25 column represents free Vit. B_{12} only. This is shown by 1) elution pattern of free Vit. B_{12} into this fraction only, 2) complete dialyzability of Vit. B_{12} contained in this fraction, 3) complete adsorption of Vit. B_{12} from this fraction by charcoal, and 4) characteristic cathodic electrophoretic mobility of Vit. B_{12} contained in this fraction. Conversely, Vit. B_{12} eluted into Peak I of the Sephadex G-25 column represents bound Vit. B_{12} only. This is evidenced by the following: 1) Radioactivity in this fraction is associated with the presence of protein (peptide) materials; 2) All Vit. B_{12} in this fraction is non-dialyzable; 3) All Vit. B_{12} in this fraction has anodic mobility only, which is characteristic for Vit. B_{12} bound to Vit. B_{12}-binders in the gastric juice. This anodic mobility cannot be due to the presence of degradation products of free Vit. B_{12}, which may also have anodic mobility(17) since these are dialyzable and have not been detected in the same batch of free Vit. B_{12} at the anodic side of electrophoretic partition.

Over 50% of the non-dialyzable Vit. B_{12}, eluted into Peak I from Sephadex G-25 column, was adsorbed by Norit A. This indicates that charcoal is well suited for adsorption of free B_{12}, but cannot be used for quantitative separation of free and bound Vit. B_{12} from mixtures containing both forms of Vit. B_{12}. This agrees with findings of Gregory and Holdsworth(18).

One of the advantages of Sephadex fractionation is the excellent yield of materials eluted from the column. We calculated the amounts of radioactivity put on and eluted from the column and noted almost 100% recovery. This cannot be duplicated by any other separation method used for assay of free and bound Vit. B_{12}. Other advantages are reproducibility, ease, and relative speed of the technic. Furthermore, Sephadex fractionation does not reduce the biological activity of materials applied. This is of significance in the work on intrinsic factor and intrinsic factor-related Vit. B_{12} binders, especially since buffers or solutions of any pH and ionic strength may be used with this column(5-7). Sephadex G-25 may be applied to the separation of free Vit. B_{12} from Vit. B_{12} bound to any biological materials such as serum, gastrointestinal secretions, and other body fluids. All these Vit. B_{12} binders have molecular weights above 3,500-4,500(9) so that they are excluded from the G-25 gel and pass into the Peak I of the effluent. This fractionation method is therefore suitable for assay of Vit. B_{12} binding capacity and biological Vit. B_{12} binders and may also substitute for dialysis or ultrafiltration methods used for this purpose.

Summary and conclusions. A good separation of bound from free Vit. B_{12} is obtained on Sephadex G-25 columns in mixtures containing free Vit. B_{12} and Vit. B_{12} bound to binders of the normal human gastric juice. Sephadex G-25 fractionation method may be very useful in the assay of Vit. B_{12} binding capacity of biological Vit. B_{12} binders and intrinsic factor materials from human and animal sources.

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Inhibition of a Human Fibrinolytic System by Normal and Pathologic Sera. (27765)

JOHN T. CORRELL* AND ALBERT SJOERDSMA

Experimental Therapeutics Branch, National Heart Institute, Bethesda, Md.

The potential importance of fibrinolytic mechanisms in health and disease has been suggested(1). However, our knowledge of fibrinolysin inhibitor concentration of serum in pathologic states is meager(2). In previous studies streptokinase was usually used in the assay system. This practice imposes a limitation since sera contain variable levels of streptokinase antibody(3).

The purpose of this study was to construct a lysing medium from human fibrinogen-plasminogen, activated with human urokinase and clotted with thrombin, and to investigate the inhibition of such a system by sera from normal human controls and from patients with various diseases. Several inhibitors to fibrinolysis have been described(2). The assay medium organized for this study was believed to contain all essential human fibrinolytic components. It was, therefore, thought that any inhibitor response measured would reflect the total inhibiting capacity of serum on all the fibrinolytic reactions, under the conditions employed. Variations in the proportions of individual inhibitors would not, of course, be observed. Measurement of the summation of serum inhibitor activity might mimic physiological conditions more realistically than would the use of purified entities. Herein is reported the fibrinolytic inhibitor activity of 16 normal human control sera and sera from 32 patients with a variety of clinical conditions.

Materials and methods. Human fibrinogen†: The solids from several vials of lyophilized, partially purified human fibrinogen were mixed intimately in a closed container. The composite analyzed 11% moisture and 80% protein; 30% of the protein was clottable. The fibrinogen was assayed for plasminogen using streptokinase as an activator and contained 0.12 caseinolytic units/mg clottable protein. The composite was stored at 4°C.

Urokinase‡: A stock solution was prepared

* Guest worker from Upjohn Co., Kalamazoo, Mich.

† Courtesy of Dr. K. B. McCall, Biologic Products Section, Michigan Dept. of Health, Lansing. Outdated material from the American Red Cross blood plasma fractionation program.

‡ Leo Pharmaceutical Products, Denmark.