

Continuous Microdetermination of Protein with a Sephadex-Copper⁶⁴ Detector Column

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The authors describe a detector column which consists of high-specific activity ⁶⁴Cu complexed with alkaline Sephadex. The protein solution to be measured is alkalinised with NaOH and pumped at constant rate through the detector column. Radioactivity in quantities equivalent to the amount of protein added is eluted and measured with a ratemeter furnished with a recorder. The method permits determination of protein in submicrogram quantities. It is insensitive to ammonia and various other small-molecular substances forming complexes with copper. A preliminary study was made on the elution properties of amino acids, peptides and various other substances and a rough estimate of the stability of the copper-Sephadex complex was obtained. In model runs, DEAE-cellulose chromatography patterns were produced for tear fluid and endolymph.

There is a need for more sensitive methods to determine total protein. To mention a few examples, when enzymatically or biologically active extracts are purified by column chromatography, the final fractions often emerge in very dilute form and require concentration before their protein content can be determined, *e.g.* by a micro-Kjeldahl method, a combination of the biuret and Folin-Ciocalteu tyrosine methods (*e.g.* Lowry¹) or the ninhydrin procedure². Also, much of the precious material is consumed by the assay. Samples of many body fluids and extracts of small organs are normally only obtainable in such minute quantities that the protein patterns of individual specimens cannot be produced.

In order to determine very small quantities of protein we have developed a "biuret" technique in which radioactive copper is bound to the protein and separated from the unbound copper by passage through Sephadex. In preliminary experiments, we found that small-molecular copper introduced onto an alkaline Sephadex column was retained in it, whereas the large-molecular copper complexes passed through³. Thus the column could be used continuously to determine protein, and there was no need to devise a cyclic system⁴ to eliminate the non-excluded small-molecular copper, which we originally

assumed would emerge from the column after the large-molecular complexes. A further improvement was to build the radiocopper into the Sephadex column before introducing the protein. When the protein sample is run through the column, an amount of radioactivity proportional to the amount of protein is eluted³.

PROCEDURE

Reagents. Copper-64. 40 mg batches of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ were irradiated for about 50 h in the maximal neutron flux ($1-2 \times 10^{12}$ n/cm².s) in the Stockholm reactor in Sweden. The experiments were begun about 4 h after the end of the irradiation, at which time the specific activity of the radiocopper was of the order of 1 C/g Cu. In our preliminary studies³, the radiocopper was purchased from the Radiochemical Centre, Amersham, England. Some irradiations have also been performed in the Finnish reactor FIR1.

Citrate-carbonate solution. 17 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ and 10 g anhydrous Na_2CO_3 made up to 100 ml.

Sephadex G-25, coarse, was purchased from AB Pharmacia, Uppsala, Sweden.

Preparation of the detector column. The Sephadex was allowed to swell overnight in 0.75 N NaOH and packed to a height of about 8 cm in a glass column 25 cm high and with an internal diameter of 8 mm. All or part of the irradiated copper salt was dissolved in 2-3 ml of distilled water and to this was added 1 ml of the citrate-carbonate solution. Most of the supernatant lye was decanted off from some of the left-over Sephadex, and about 10 ml of the half-dry alkaline gel plus a few ml of the lye were agitated with the radiocopper-citrate-carbonate solution in a small Erlenmeyer flask. Agitation was continued until the blue colour disappeared from the supernatant and was adsorbed on the Sephadex. The radiocopper-Sephadex ("blue Sephadex") was packed on the top of the Sephadex column, 0.75 N NaOH solution being used for suspension purposes. The column was washed with NaOH or alkalinised effluent from the chromatography column subsequently to be connected to the detector column until the radioactivity eluted reached a steady value (3-15 times the normal background count, depending on the amount of ⁶⁴Cu added and the rate of flow).

Radioactivity counting. ⁶⁴Cu emits gamma (0.6 %), beta (38 %) and positron (19 %) radiation. Many counting procedures may therefore be devised, some of which should be highly efficient and give low background counts, e.g. coincidence counting and pulse-height analysis. Owing to the costs involved, the relative merits of the different procedures have not yet been explored. Most of our data have been obtained with the following simple procedure: The effluent from the detector column (shielded with lead) is led through a glass spiral kept in the well of an EKCO type N597 sodium iodide scintillation crystal. The radioactivity is registered with the aid of a ratemeter coupled to a recorder (Fig. 1). In some experiments, in order to keep the readings obtained from unknown samples within the scale limits, the output of the ratemeter was made logarithmic. Since the half-life of the isotope is short (12.8 h), accurate timing of the procedure is important, and the radioactivity counts have to be multiplied by decay correction factors.

Alkalinisation of the chromatographic effluents. This was achieved with the aid of a Technicon AutoAnalyzer multichannel peristaltic pump. The effluent and the sodium hydroxide, of a normality sufficient to bring the pH to 13 or more (usually 3 N), were both metered at 0.34 ml/min, Manifold tubing with an inner diameter of 0.03 inch being employed. An AutoAnalyzer mixing coil (a horizontal glass spiral) was inserted between the pump and the detector column.

Chromatographic procedure. Chromatographic fractionations were performed only to illustrate the use of the detector system and no attempts were made to perfect the fractionation procedure to suit the mixtures under study. The DEAE-cellulose system in use in our laboratory⁵ was employed but with the dimensions reduced. The chromatographic column (19 cm long, 6 mm inner diameter) was filled with Schleicher & Schüll DEAE-cellulose. Elution was achieved by applying continuously mixing phosphate buffers, which produced an increasing concentration (5 mM to 0.5 M) and falling pH (7.5 to 3) gradient.

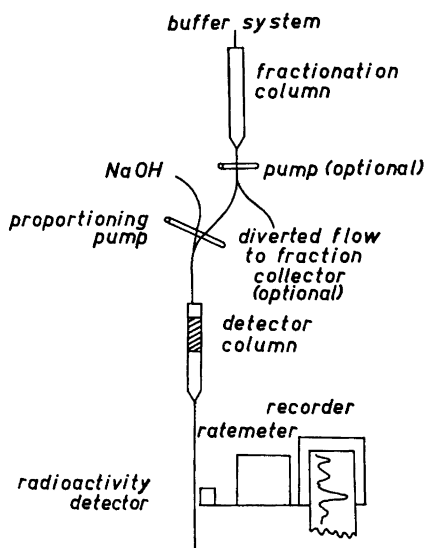


Fig. 1. The detector system.

When most of the chromatography effluent is to be used for other purposes, only part of the effluent from the fractionation column needs to be fed into the detector. A higher flow rate in the fractionation system is achieved by inserting a third, thicker manifold tube into the peristaltic pump. If pulsating movements in the chromatographic column are to be avoided, a separate continuous flow pump may be used for this purpose.

The principle of the system is illustrated in Fig. 1.

Materials fractionated by chromatography. Tear fluid was obtained from healthy subjects by bromoacetone irritation. Material obtained from several persons was pooled and frozen. A precipitate formed when the pool was thawed and was removed by centrifugation.

Fluid from the inner ear was obtained at necropsy. The semicircular canals were exposed on both sides, a thin needle was inserted and the clear liquid — probably endolymph — was withdrawn. Altogether about 0.03 ml was obtained, and it was immediately diluted with the 5 mM phosphate buffer used in the chromatography.

RESULTS

A straight-line relationship between the amount of protein fed into the column and the eluted radioactivity counts corrected for radioactive decay was observed from the macro-range, where the emerging copper complex could be determined colorimetrically, down to the minimum value detectable. In our system, 0.12 μg of bovine albumin introduced in 1 ml volume still elicited noticeable recorder deflections. The accuracy of the method is extremely good in the higher ranges; in the 1 μg range the standard deviation of the points from the standard curve was 4.9%. Probably this value can be improved, especially by increasing the specific activity of the isotope and the sensitivity of the counting procedure.

Since the column is to be used for the registration of column effluents, various buffers and salt solution used in chromatographic work were alkalined and fed into the detector column (Table 1). It is seen that low background

Table 1. Effects on the detector column of various solutions used in column chromatographic procedures. The solutions were alkalised to pH 13 with concentrated NaOH and run through the column continuously. Concentrations 0.5 M unless otherwise stated.

- A. Do not elute significant amounts of ^{64}Cu :
 ammonia, sodium chloride, phosphate, acetate, diethylbarbiturate (0.05 M)
- B. Elute ^{64}Cu :
 glycine, citrate, borate, tris(hydroxymethyl)aminomethane

Table 2. Elution properties of various substances fed into the detector column intermittently in small doses. Dose 60 μg in 1 ml volume unless otherwise stated.

- A. Do not elute ^{64}Cu :
 alanine, arginine, aspartic acid, asparagine, cystine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine
 glycyl-glycine, glycyl-tyrosine, gramicidin, cycloserine, histamine
 urea, biuret, ethylenediamine, 2,2'-dipyridyl, salicylic acid, boric acid (2 mg), citric acid (2 mg)
- B. Elute ^{64}Cu :
 cysteine, histidyl-histidine, glycyl-glycyl-glycine, leucyl-glycyl-glycine, glycyl-glycyl-glycyl-glycine, glycyl-glycyl-glycyl-glycyl-glycine-ethyl ester, ethylenediaminetetraacetic acid, tris(hydroxymethyl)aminomethane, gluconic acid

radioactivity is eluted by sodium chloride, acetate, phosphate, diethylbarbiturate and ammonia. We also checked that protein elutes ^{64}Cu when administered in these media. By contrast, borate, "tris", glycine and citrate buffers proved to be unsuitable protein solvents.

In protein chemical work it is of interest to know whether amino acids and peptides emerging from the fractionation column elute radioactivity. Accordingly, a number of substances at hand were tried. As seen from Table 2, the only amino acid to elute radioactivity when fed in small intermittent doses was cysteine, no doubt owing to mercaptide formation. Among the dipeptides, histidyl-histidine eluted radiocopper. The longer peptides all eluted radiocopper, except gramicidin.

In Table 2 are also tabulated various other substances, some of which were included because their Cu^{2+} complexes had known stability constants. It is noteworthy that citrate, which has the logarithmic stability constant 14.21 ($K_{\text{MHL}}^{\text{M}}$)⁶, does not elute radioactivity, whereas gluconic acid and EDTA do ($\log K_1$ 18.29 and 18.80, respectively)⁶. In experiments on the macroscale the interesting observation was made that biuret (allophanamide, carbamyl-urea) runs freely through alkaline Sephadex but is retained in the copper-Sephadex column, which acquires a violet hue.

To demonstrate the sensitivity of the detector system, model runs were performed with tear fluid and endolymph. As illustrated by Figs. 2 and 3, satisfactory recorder deflections were obtained with the extremely small sample of endolymph, containing about 200 μg of protein (relative to a bovine albumin standard) and one drop (0.05 ml) of pooled tear fluid, containing about 350 μg of protein. The tear pattern agrees with that obtained by paper electrophoresis⁷.

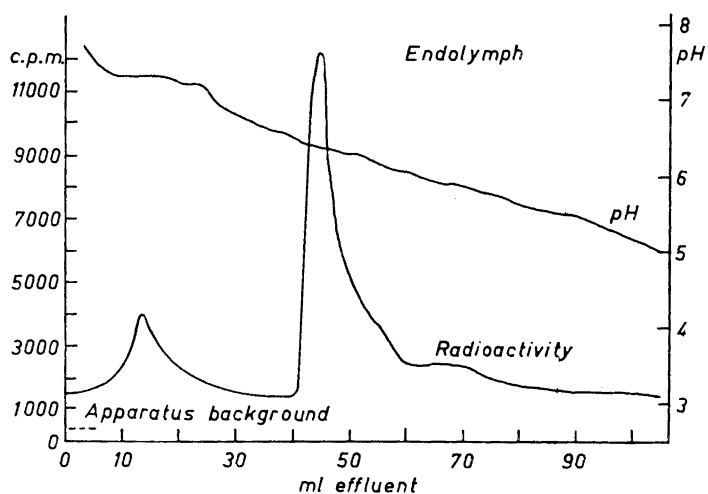


Fig. 2. Fractionation of fluid from the inner ear ("endolymph") on a DEAE-cellulose column, the effluent of which was registered with the detector system. The sample contained about 200 μg of protein.

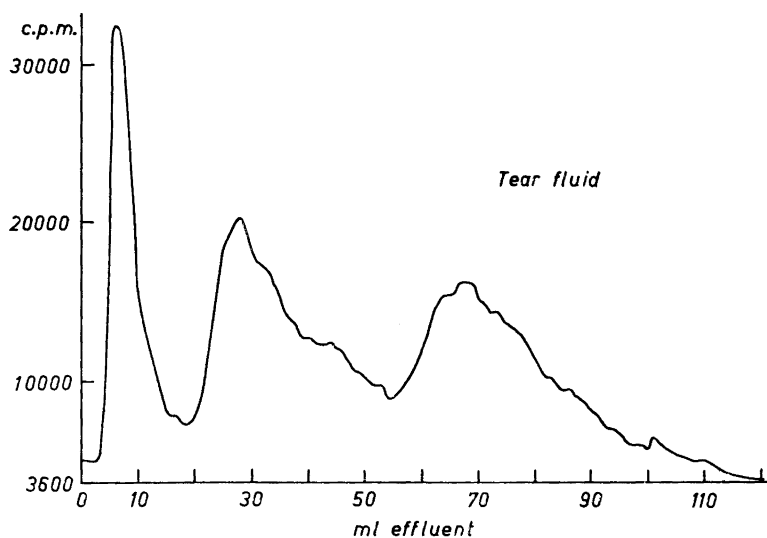


Fig. 3. Model run in which one drop (0.05 ml) of tear fluid was fractionated on a DEAF-cellulose column. About 350 μg of protein was contained in the sample.

DISCUSSION

It seems evident that the "blue Sephadex" consists of a copper-Sephadex complex. It is fairly stable; the alkaline gel takes up copper even from its citrate complex. Its stability constant may be assumed to lie between those of copper citrate and gluconate. These data can be used to predict the elution properties of additional substances.

However, it should be noted that in the detector column there is an excess of unbound Sephadex, which owing to mass action may detach the copper from more stable complexes running through in small quantities. Similar considerations explain why citrate and glycinate ($\log K_1$ about 8.2)⁶, run through intermittently in small doses, do not elute radioactivity, but dissolve radiocopper when applied continuously.

The stability constant of the copper-protein complex which remains after passage through the column must be fairly high. This is also shown by the finding that the violet biuret colour is formed in the ordinary biuret reaction when the copper is added to the protein in the form of versenate⁸, which has the stability constant 18.80⁶. Our preliminary results with the peptides lead us to infer that at least three nitrogens *plus* a fourth nitrogen or a carboxyl in suitable steric arrangement in the molecule are required to give the stable protein-copper complex, which is in accordance with the general view⁸⁻¹⁰ on the structure of the "biuret" compound. Steric hindrance probably explains why the cyclic peptide gramicidin does not elute radioactivity.

Experiments on the macroscale showed that biuret itself is retained in the detector column, which turns violet. Probably a mixed biuret-copper-Sephadex complex is formed with a structure resembling the ammonia-copper-cellulose complexes in a solution of cellulose in Schweitzer's reagent¹¹.

As far as we know, the present method is the most sensitive one devised for the quantitative determination of protein. With the aid of refined radioactivity measurement and higher specific activity of the copper isotope it should be possible to reduce the lower limit of the method by a factor of perhaps 10 or even more. More important, our method makes possible the column chromatographic, electrophoretic or gel filtration analysis of materials which have been more or less inaccessible to such study owing to their small amounts. Compared with the biuret-Folin-Ciocalteu method (Lowry¹ and others), an obvious advantage is the linear relation between the reading and the amount of protein in the sample. The Lowry method is sensitive to changes in pH², while in the present method the extremely alkaline pH used is easily produced. An obvious advantage over the micro-Kjeldahl and ninhydrin methods is that the radiocopper technique can easily be utilised for continuous measurement of column effluents and that it is insensitive to ammonia. If a larger column is used for the fractionation, only a small fraction of the effluent is needed for the determination and thus very little material is consumed. The saving of material is almost as great as when ultraviolet light absorption is measured, a method which is less sensitive and less specific.

The main drawback of our method is the short half-life of the ⁶⁴Cu isotope. Provided a reactor is near, it can easily be prepared by irradiating copper nitrate with neutrons and no radiochemical preparations are required. A

longer-lived isotope, ^{67}Cu , exists, but is not commercially available. On the macroscale our system also works with nickel, but the ^{63}Ni which is commercially available emits only weak beta radiation and has a very long half-life, which precludes the preparation of high-specific activity material. The other metals tried gave disappointing results.

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