

# A General Method for the Assay of Binders. Application to the Potentiometric Determination of Albumin in Human Serum, Plasma and Whole Blood<sup>1</sup>

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From the generalized theory of binding (stepwise equilibrium model, Scatchard model) it can be derived that in a solution containing the binder and a specific ligand, the bound ligand concentration is linearly related to the total binder concentration only if the free ligand concentration is constant. We designed a general method which is based on that principle and we have shown its validity for the albumin assay in serum, plasma and whole blood. We have tested the ligands bromocresol purple and picrate and used ligand-ion selective electrodes to monitor free ligand concentration in a homogeneous solution. The method has excellent linearity. We chose picrate (PIC) as ligand, because of better specificity. Comparison with the bromocresol purple (BCP) and bromocresol green (BCG) spectrophotometric procedures for 93 samples gave the regression equations:  $y(\text{PIC}) = 0.98x(\text{BCP}) + 0.76$  (g/L,  $r = 0.979$ ) and  $y(\text{PIC}) = 0.95x(\text{BCG}) - 1.3$  ( $r = 0.947$ ), respectively. Within-day and day-to-day precision was up to 1.5% and 2.3%, respectively. Results for albumin in plasma, when transformed to expected whole blood values, were in close agreement with those obtained by direct analysis of whole blood (in the presence of erythrocytes).

**KEY WORDS:** Serum albumin; blood proteins; electrodes, ion-selective; ligands; potentiometry.

The reversible binding of a ligand to a macromolecule (binder) is an ubiquitous phenomenon of importance to a large number of scientific disciplines, e.g. endocrinology, immunology, pharmacology, enzymology, protein chemistry and clinical chemistry. Binder-ligand interactions can be extremely strong, such as antigen-antibody interactions, or the binding of biotin to avidin. Weaker interactions include substrate binding to enzymes, oxygen binding to hemoglobin, drug binding to receptors, cAMP binding to the regulatory subunit of protein kinases and steroid hormone binding to serum proteins or to specific cytoplasmic receptors. Steroid hormone-receptor complexes with specific regions of the genome may also be considered as ligand-macromolecule interactions.

In certain clinical conditions, the quantitation of binder concentration is useful in the diagnosis and investigation of disease. Such examples include the assays of LDL-receptor or insulin receptor concentrations for the investigation of essential familial hyper-

cholesterolemia and type II diabetes mellitus, respectively. The direct or indirect assay of thyroid hormone binding globulin (TBG) and the assay of transferrin (TIBC) are examples of the assay of binders in the routine clinical chemistry laboratory.

Albumin is probably the best known binder of biological fluids (1). This protein binds, generally with low affinity, endogenous and exogenous molecules like thyroid or steroid hormones, bilirubin, free fatty acids, metals and drugs (2, 3).

The binder concentration of a sample can be determined by two general procedures: (a) By immunological methods with use of specific antibodies directed against antigenic determinants of the binder (4); in this case, enzyme or fluorescence immunoassays, radioimmunoassays (RIA) or immunoradiometric assays (IRMA) can be used. (b) With procedures which use specific ligands; in this case, the measurement of the bound or free ligand can be used indirectly to determine the binder concentration in the sample. Examples of this approach include the determination of  $T_3$ -uptake ( $T_3$ -U, indirect assay of TBG), the measurement of total iron binding capacity (TIBC, indirect assay of transferrin) and the assay of albumin in human serum by dye-binding techniques. The latter technique is homogeneous, that is, the bound ligand concentration can be measured directly because the dye binding to albumin causes a change in absorbance of the solution. In most instances (e.g. in the  $T_3$ -U or TIBC assay or in RIA) the bound and free ligand must be separated before the quantitation of either.

Albumin in biological fluids can be determined with immunological procedures like electroimmunoassay (5), radial immunodiffusion (6), immunonephelometry (7) and radioimmunoassay (8). The methods are specific and sensitive but they are not suitable for routine use because they are slow, costly, and difficult to automate. In most clinical chemistry laboratories, albumin is measured routinely with dye-binding techniques. Of the albumin ligands used in the past, bromocresol green (BCG) remains the most popular (9). However, because of the well-established binding of BCG to other serum proteins (10, 11) and the positive bias of these procedures, other binders were tested to increase specificity. It has been shown that bromocresol purple (BCP) is a specific ligand for albumin and that BCP procedures give results significantly lower than those of BCG

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methods and correlate well with specific immunological albumin assays (12, 13).

In this paper, we describe the development of a new general method for the assay of binders. It is based on the binding of a specific ligand. In contrast to other techniques where the total ligand concentration remains constant, in our method the free ligand concentration is set at a constant preselected level.

The method was applied to the determination of albumin in serum, plasma and whole blood. The specific ligand used was picrate. BCP was found unsatisfactory for this application because by potentiometry, this ligand gives results with positive bias. A picrate ion-selective electrode was used to monitor the free picrate concentration continuously in the reaction mixture.

The albumin potentiometric assay was found to be simple, sensitive, specific and precise. To our knowledge it is the first albumin method that performs equally well for serum, plasma and whole blood. The ability to measure constituents in whole blood in the presence of red blood cells may prove useful in the future, since bed-side monitoring is gaining popularity and acceptance in the field of clinical chemistry. This ability derives from the use of ion-selective electrodes as transducers in our technique.

**Theoretical background**

Two general mathematical approaches are widely used for the quantitative description of the multiple equilibria taking place when a molecule B (binder), with *n* binding sites, binds reversibly a ligand molecule, L. One of these, called the stepwise equilibrium model or thermodynamic treatment (14), assumes that the molecule B may bind up to *n* ligand molecules in a sequence of *n* stepwise reactions and applies the mass action law for each reaction. The concentration *C<sub>b</sub>* of the bound ligand is then given by the following equation:

$$C_b = \frac{K_1 C_f + 2K_1 K_2 C_f^2 + \dots + nK_1 K_2 \dots K_n C_f^n}{1 + K_1 C_f + K_1 K_2 C_f^2 + \dots + K_1 K_2 \dots K_n C_f^n} \times B_t = \frac{\sum_{i=1}^n i \left(\prod_{k=1}^i K_k\right) C_f^i}{1 + \sum_{i=1}^n \left(\prod_{k=1}^i K_k\right) C_f^i} B_t \dots \dots \dots [1]$$

where *C<sub>f</sub>* is the free (unbound) ligand concentration, *B<sub>t</sub>* is the total concentration of the binder and *K<sub>1</sub>, K<sub>2</sub>, . . . , K<sub>n</sub>* are the stoichiometric binding constants.

The second approach known as the Scatchard model (15), in contrast to the stepwise equilibrium model, focuses attention on the individual binding sites of the binder and applies the law of mass action for each site, defining the site equilibrium constant, and assuming that the affinity of each particular site for the ligand is not influenced by the extent of occupancy of the other sites. In addition, this model groups the *n* binding sites into *m* distinct classes. The class *i*, contains a number *n<sub>i</sub>* of individual sites that have the same affinity for the ligand. The site binding constant *k<sub>i</sub>* (intrinsic binding constant) characterizes the sites of the *i*th class. The concentration *C<sub>b</sub>* of the bound ligand is given by the

well known equation:

$$C_b = \left( \frac{n_1 k_1 C_f}{1 + k_1 C_f} + \frac{n_2 k_2 C_f}{1 + k_2 C_f} + \dots + \frac{n_m k_m C_f}{1 + k_m C_f} \right) \times B_t = \sum_{i=1}^m \frac{n_i k_i C_f}{1 + k_i C_f} B_t \dots \dots \dots [2]$$

The stepwise equilibrium model is more general than the Scatchard model and applies to all multiple equilibrium reactions independently of mechanism. Under certain conditions the two models are equivalent and the constants of the stepwise equilibrium model can be approximated from those of the Scatchard model (16, 17).

From either Equation 1 or 2 we observe that regardless of the model used and of the number of binding sites on the binder, the concentration of bound ligand is linearly related with the total concentration of the binder in the solution, provided that the free ligand concentration remains constant. Therefore, if the *C<sub>f</sub>* is constant we can write Equations 1 and 2 in the simple form:

$$C_b = F \cdot B_t \dots \dots \dots [3]$$

where *F* is a constant term. The various analytical methods used to determine binders, e.g. the spectrophotometric dye binding methods, have been designed in such a way that the total rather than the free ligand concentration remains constant. Under such conditions the linearity of the calibration graphs is limited.

In our method, we chose to maintain the free ligand concentration constant. We used ion-selective electrodes (ISE) as transducers, because of their attractive property to respond directly to free ion activity. So, in a solution containing the binder and an ionic ligand, the free ligand can be monitored continuously with a ligand-sensitive ISE according to the Nernst equation:

$$E = E_0 + S \log C_f \dots \dots \dots [4]$$

where *E* is the electrode potential, *E<sub>0</sub>* is a constant term if the measurements are carried out in dilute solutions (i.e., at concentrations <10<sup>-2</sup> mol/L) or at constant ionic strength, and *S* is the electrode slope which is found experimentally from a calibration graph.

**Principle and formulas**

The principle of the method is illustrated graphically in Figure 1.

Suppose that a solution contains a certain concentration of an ionic ligand. Then a ligand ISE generates a stable potential (EMF) vs a reference electrode. If a sample containing the binder is added, a fraction of the ligand will be bound, the free ligand concentration will decrease and the EMF will change. We bring the free ligand concentration back to its initial level, by adding aliquots of another more concentrated ligand solution, until the EMF reaches its initial value. At this point, according to Equation 3, the bound ligand concentration is proportional to the final binder concentration in the solution, because the free ligand concentration is kept constant independently of the binder concentration. Also, it can be proved that the volume of the con-

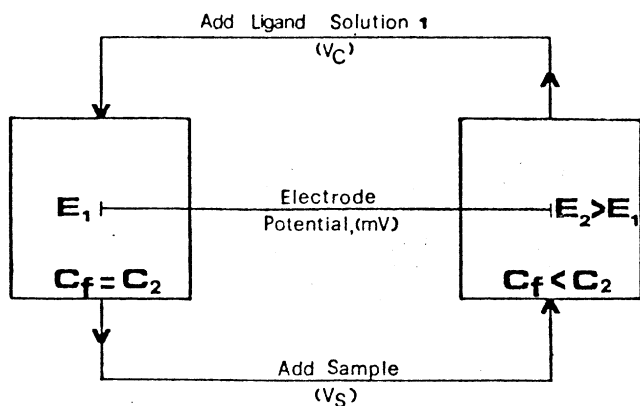


Figure 1 — Schematic representation of the principle of the proposed potentiometric assay. Addition of a sample volume  $V_s$  into the measuring cell containing the electrodes and a ligand solution of concentration  $C_f$  causes the EMF to increase from  $E_1$  to  $E_2$ . The potential is readjusted to  $E_1$  by adding a volume  $V_c$  of a concentrated ligand solution 1.  $V_c$  is linearly related to the albumin concentration of the sample. For the picrate method  $C_f = C_2 = 3.26 \times 10^{-5}$  mol/L and solution 1 is  $2.04 \times 10^{-3}$  mol/L.

concentrated ligand solution consumed is a linear function of the binder concentration in the sample.

Two ligand solutions, 1 and 2, are prepared. Solution 2 results from the dilution of solution 1 by a factor of  $D$  so that,

$$C_1 = D \cdot C_2 \dots\dots\dots [5]$$

An aliquot,  $V_0$ , of solution 2 is pipetted into a measuring cell containing the electrodes. The EMF,  $E_1$ , of the ligand ISE is recorded. A volume,  $V_s$ , of sample or standard of concentration  $C_s$  is injected. The EMF changes to a new value  $E_2$ . An appropriate volume,  $V_c$ , of solution 1 is then added so as to adjust the EMF to its initial value  $E_1$  ( $\pm 0.1$  mV). At this point, the free ligand concentration,  $C_f$ , equals  $C_2$ . The total ligand concentration  $C_t$  is given by the equation:

$$C_t = \frac{V_0 C_2 + V_c C_1}{V_0 + V_s + V_c} \dots\dots\dots [6]$$

The total binder concentration,  $B_t$ , is:

$$B_t = \frac{V_s C_s}{V_0 + V_s + V_c} \dots\dots\dots [7]$$

Conservation of the ligand in solution requires that:

$$C_t = C_b + C_f \dots\dots\dots [8]$$

Substituting  $C_b$  and  $C_t$  from Equations [3] and [6] into Equation [8] yields:

$$\frac{V_0 C_2 + V_c C_1}{V_0 + V_s + V_c} = F B_t + C_f \dots\dots\dots [9]$$

After substituting the  $B_t$  from Equation [7] into Equation [9] and rearrangement, yields ( $C_f = C_2$ ):

$$V_c C_1 - V_c C_f - V_s C_f = F C_s V_s \dots\dots\dots [10]$$

Combining Equations [5] and [10] yields:

$$V_c D C_f - V_c C_f - V_s C_f = F V_s C_s \dots\dots\dots [11]$$

and

$$(D - 1) V_c - V_s = (F V_s / C_f) C_s \dots\dots\dots [12]$$

If we substitute the term  $(D - 1) V_c - V_s$  with  $Y$  and  $(F V_s / C_f)$  with  $A$ , then Equation [12] becomes:

$$Y = A C_s \dots\dots\dots [13]$$

which is the equation of the calibration curve.

**Apparatus**

Potentiometric measurements were made at room temperature ( $24 \pm 1^\circ\text{C}$ ) with a Corning Research pH/mV meter (Model 12) using the bromcresol purple (BCP) or picrate anion selective electrodes in conjunction with a calomel reference electrode. The pH measurements were carried out with a combined glass-calomel electrode (Radiometer, Copenhagen, Denmark). Absorbances were measured with a Hitachi Perkin-Elmer double beam spectrophotometer.

**Reagents**

All solutions were prepared in deionized distilled water from analytical grade materials, unless otherwise stated.

**BUFFERS**

Citrate, pH 2.5 and 3.0; acetate, pH 4.0, 4.4, 4.8 and 5.2; phosphate, pH 7.0; and Tris, pH 8.0. Concentrations of all buffers were 0.10 mol/L and were prepared from the corresponding acid or acid salt and 18 mol/L NaOH solution.

**ISOTONIC PHOSPHATE BUFFER**

Dissolve 2.433 g of NaCl in 1 L of 0.1 mol/L phosphate buffer. The total osmolality was found to be 294 mosm/Kg.

**ISOTONIC TRIS BUFFER**

Dissolve 4.100 g of NaCl in 1 L of 0.1 mol/L Tris buffer. The total osmolality was 294 mosm/Kg.

**BCP SOLUTION 1 ( $4 \times 10^{-3}$  MOL/L)**

Dissolve 0.225 g of bromcresol purple (sodium salt, Fluka) in 100 mL of water.

**BCP SOLUTION 2**

This solution contained bromcresol purple (40  $\mu\text{mol/L}$ ) and Brij-35 (0.25 g/L) in acetate buffer (0.10 mol/L, pH 5.2). It was prepared according to Pinnel and Northam (13).

**PICRATE SOLUTIONS 1 AND 3 ( $2.04 \times 10^{-3}$  MOL/L)**

Dissolve 0.234 g of picric acid in 500 mL of either phosphate buffer (for solution 1) or isotonic Tris buffer (for solution 3).

PICRATE SOLUTION 2 ( $3.26 \times 10^{-5}$  MOL/L, pH 7.0)

A volume of 16.00 mL of picrate solution 1 is diluted to 1 L with the phosphate buffer (dilution factor,  $D = 62.5$ ).

PICRATE SOLUTION 4 ( $3.26 \times 10^{-5}$  MOL/L, pH 8.0, ISOTONIC)

A volume of 16.00 mL of picrate solution 3 is diluted to 1 L with isotonic Tris buffer ( $D = 62.5$ ).

PICRATE SOLUTION 5 ( $3.26 \times 10^{-5}$  MOL/L, pH 7.0, ISOTONIC)

A volume of 16.00 mL of picrate solution 1 is diluted to 1 L with isotonic phosphate buffer ( $D = 62.5$ ).

## STANDARD HUMAN SERUM ALBUMIN (HSA) SOLUTIONS

These were prepared by weighing the appropriate quantity of human serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO 63178) and dissolving it in water. We standardized these solutions by using the bromcresol purple spectrophotometric method (see Procedures) and the biuret method (18) with a 3 g/L human albumin/total protein standard of Gilford Diagnostics (Irvine, CA 92714).

## Procedures

## PICRATE-ION SELECTIVE LIQUID MEMBRANE ELECTRODE

Preparation of the liquid ion-exchanger and characteristics of the picrate-ion selective electrode have been described elsewhere (19). The liquid ion-exchanger consists of the tetrapentylammonium-picrate ion pair, dissolved in 2-nitrotoluene (0.01 mol/L). An Orion (Cambridge, MA 02139) liquid membrane electrode body (Model 92) was used as the electrode assembly with the Millipore LCWPO 1300 PTFE porous membrane. The PTFE membrane was cut to the appropriate size and a stack of four was used to separate the organic phase from the test solution. The internal reference solution was 0.1 mol/L NaCl — 0.01 mol/L sodium picrate (saturated with silver chloride). When not in use, the electrode was kept in a  $10^{-2}$  mol/L solution of sodium picrate.

## BROMCRESOL PURPLE — ANION SELECTIVE, POLY(VINYL-CHLORIDE) MATRIX, MEMBRANE ELECTRODE

The construction and analytical characteristics of this new electrode and its application to the study of the BCP — albumin interaction, will be described elsewhere.

## POTENTIOMETRIC DETERMINATION OF HSA IN SERUM AND PLASMA

## 1. Use of BCP anion as ligand (BCP-pot method)

Pipet 10.00 mL of the BCP solution 2 into a beaker,

immerse the BCP-ISE and the reference electrode in the solution, start stirring and read the potential after stabilization to  $\pm 0.1$  mV ( $\sim 30$  s). Add 50  $\mu$ L of serum or plasma sample or aqueous standard HSA solution. The binding of the BCP anion to albumin causes an instantaneous increase of the electrode potential. Bring the potential back to its initial value by adding small volumes of the BCP solution 1. Record the total volume,  $V_c$ , consumed and use this value for the calculations.

## 2. Use of picrate anion as ligand (PIC method)

Pipet 2.00 mL of the picrate solution 2 into a small beaker. Immerse the picrate ISE and the reference electrode in the solution, start stirring and read the potential after stabilization to  $\pm 0.1$  mV ( $\sim 30$  s). Add 50  $\mu$ L of serum or plasma sample or standard HSA solution. The binding of the picrate anion to albumin causes an instantaneous increase of the potential. Add small volumes of the picrate solution 1 to adjust the potential to its initial value. Record the total volume,  $V_c$ , consumed and use this value for the calculations.

## POTENTIOMETRIC ASSAY OF ALBUMIN IN WHOLE BLOOD USING THE PICRATE ANION AS LIGAND

The procedure is the same as for serum albumin determination (PIC method, procedure 2) except that picrate solutions 3 and 4 are used instead of solutions 1 and 2, respectively, and 100  $\mu$ L of well-mixed heparinized whole blood is used as sample. For the calibration curve, 50  $\mu$ L of HSA standards is used.

## SERUM ALBUMIN ASSAY BY THE BCG SPECTROPHOTOMETRIC METHOD (BCG METHOD)

The method proposed by Peters *et al.* (18) was applied using the HSA standards described above. The absorbance was measured at 628 nm with a delay time of 30 s after mixing the reagent with the HSA standard or serum sample.

## SERUM ALBUMIN ASSAY BY THE BCP SPECTROPHOTOMETRIC METHOD (BCP-SPEC METHOD)

Pipet 5.0 mL of the BCP solution 2 into a test tube. Add 30  $\mu$ L of serum sample or HSA standard, mix with a rotation mixer and read the absorbance at 600 nm.

## HEMATOCRIT (HCT) VALUE DETERMINATIONS

These were carried out with the macromethod proposed by Rice (20).

## Calculations

## CALIBRATION CURVE

From the volume,  $V_c$ , consumed for each standard (in  $\mu$ L) calculate the quantity  $Y = 61.5 V_c - 50$ , where 50 is the value of  $V_c$ , in  $\mu$ L. Plot  $Y$  vs  $C_s$  (concentration of albumin in the standards) to obtain the calibration curve.

TABLE 2  
Determination of Albumin in Ten Serum Samples by Four Different Methods

Serum Sample	Spectrophotometry		Potentiometry		Absolute errors (g/L)	
	(g/L)		(g/L)		BCPpot vs BCPspec	PICpot vs BCPspec
	BCP	BCG	BCP	PIC		
1	13.5	21.1	23.2	13.7	+9.7	+0.2
2	16.6	18.8	23.7	15.1	+7.1	-1.5
3	27.2	31.1	38.2	29.0	+11.0	+1.8
4	27.3	32.8	35.5	27.4	+8.2	+0.1
5	30.0	33.9	41.5	29.7	+11.5	-0.3
6	31.5	34.3	44.8	33.5	+13.3	+2.0
7	34.0	35.8	47.6	32.5	+13.6	-1.5
8	41.7	44.7	55.5	42.6	+13.8	+0.9
9	43.0	44.7	53.6	43.5	+10.6	+0.5
10	43.8	46.5	63.5	43.5	+19.7	-0.3

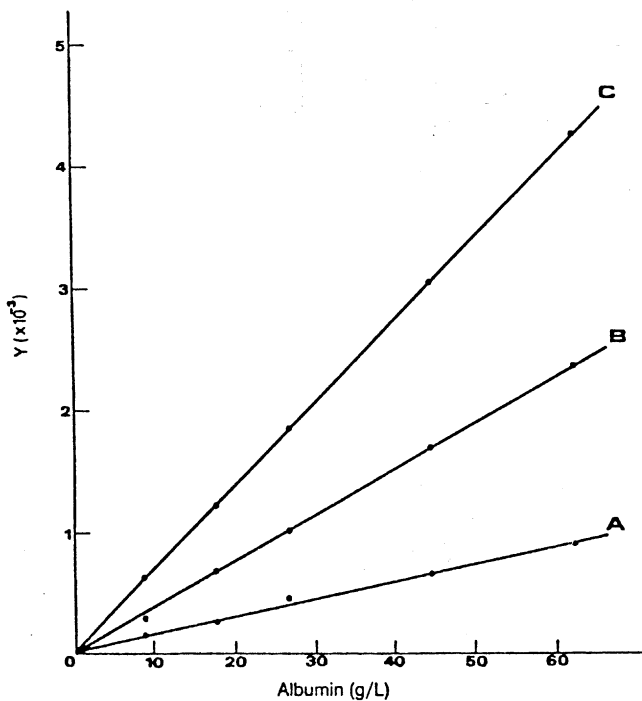


Figure 4 — Standard curves for the assay of albumin at pH 7.0 (phosphate) with picrate as ligand, at three levels of free picrate concentration. (A)  $3.26 \times 10^{-4}$  mol/L, (B)  $8.00 \times 10^{-5}$  mol/L, (C)  $3.26 \times 10^{-5}$  mol/L.

#### USE OF PICRATE ANION AS LIGAND

In Figure 4, standard curves at three levels of free picrate concentration, at pH 7.0, are presented. As shown for BCP, the slope increases as the free picrate concentration decreases. The effect of pH was examined in the range from 2.5 to 8 at constant free picrate concentration,  $3.26 \times 10^{-5}$  mol/L (Figure 5). The slope of the calibration curve is greater in acetate buffer of pH 4.0 than in citrate buffer of pH 2.5. At higher pH, the slope decreases significantly.

#### Serum assays

##### CHOICE OF THE LIGAND

In order to select which of the two ligands (BCP or

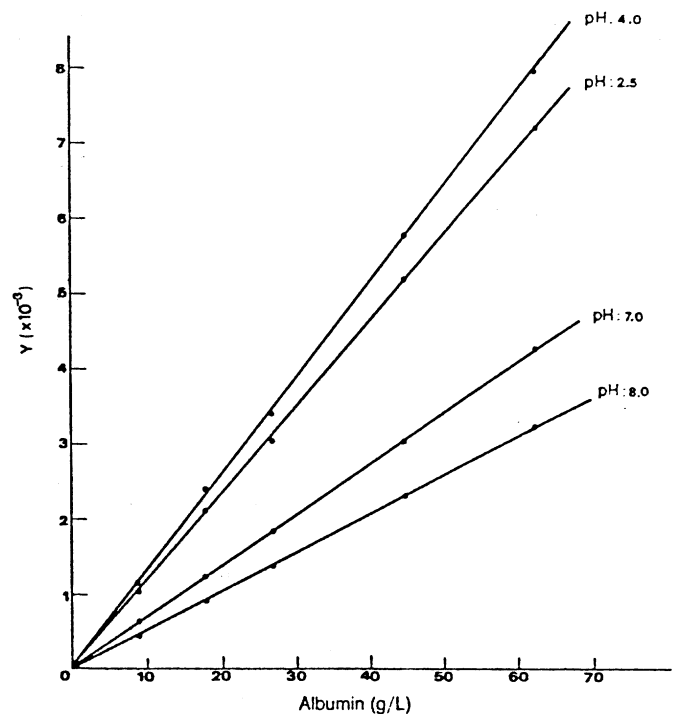


Figure 5 — Effect of pH on the standard curve of the albumin assay using picrate as ligand. pH 2.5 (citrate) pH 4.0 (acetate), pH 7.0 (phosphate), pH 8.0 (Isotonic Tris buffer).  $C_f = 3.26 \times 10^{-5}$  mol/L. The standard curves at pH 7.0 and 8.0 are representative of the assay in serum and whole blood, respectively.

picrate) is more suitable for serum assays, we determined the albumin concentration in ten serum samples from normal individuals and patients with nephrotic syndrome, by four different methods, i.e., the manual BCP and BCG spectrophotometric methods and the proposed potentiometric method using BCP or picrate as ligands (see Procedures). The results are presented in Table 2. Assuming that the BCP-spec method is specific for albumin, we observe that (i) the BCG assay has a positive bias, (ii) the BCP-pot assay exhibits a significant positive bias greater than that of the BCG assay and (iii) the PIC-pot assay gives results in close agreement with the BCP-spec assay. In the last two columns of Table 2, the absolute errors of the two potentiometric

TABLE 3  
Precision Studies for Serum Albumin Assays

Mean (g/L)	SD (g/L)	CV (%)
Within day (n = 10)		
15.0	0.2	1.43
32.6	0.5	1.53
52.7	0.6	1.14
Day-to-day (n = 10)		
14.9	0.4	2.27
32.9	0.5	1.63
52.4	0.8	1.61

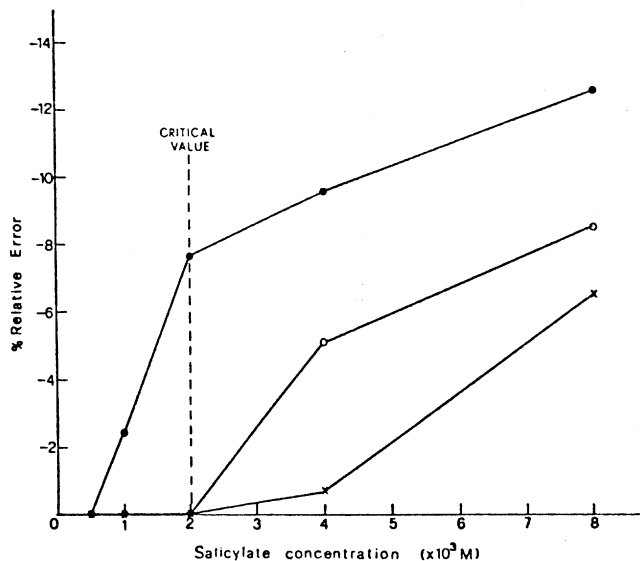


Figure 6 — Salicylate interference expressed as percent negative relative error vs salicylate concentration at three levels of albumin: (●) 52.7 g/L (○) 32.6 g/L (×) 15.0 g/L. The critical value refers to the upper therapeutic level of the drug in serum.

methods vs the BCP spectrophotometric method are presented.

When the determinations were carried out at lower pH than those suggested under Procedures, the bias of the BCP-pot method increased. At pH 4.0 a small positive bias is also detectable with the PIC-pot method.

After these observations we chose the picrate anion as ligand. Although low pH values gave calibration curves with greater slopes (better sensitivity, Figure 5) we chose the pH of 7.0 for the assay because at this pH we found the most suitable compromise between sensitivity and specificity effects.

INTERFERENCES

Two icteric sera with bilirubin concentrations of 99 and 127 μmol/L were analyzed by both the PIC-method and the BCP-spec method. Albumin concentrations were 11.9 and 21.1 g/L by the PIC method, and 12.0 and 21.3 g/L by the BCP method.

Salicylate interference in the range 0 to 8 × 10<sup>-3</sup> mol/L was tested by measuring the albumin concentration in three serum pools before and after the addition

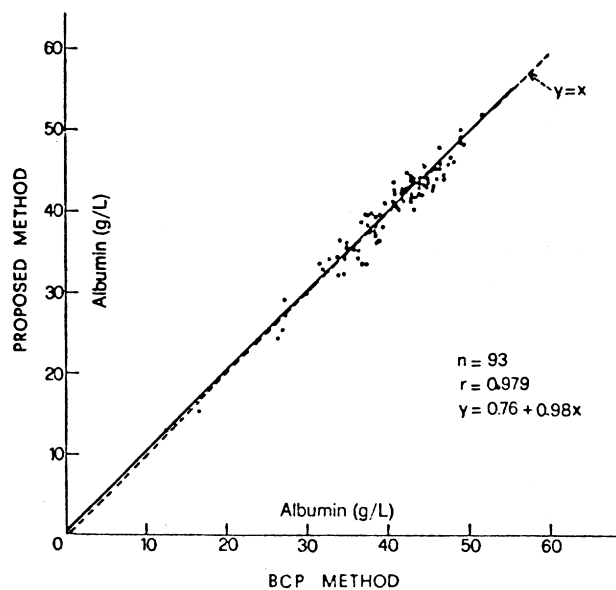


Figure 7 — Correlation between the proposed potentiometric assay (picrate as ligand) and the BCP spectrophotometric procedure. Broken line indicates the ideal situation where  $y = x$ .

of aliquots of a 0.1 mol/L aqueous sodium salicylate solution. In Figure 6, the % relative error (negative) in the albumin determination as a function of the salicylate concentration in the sample, is presented. We observe that the higher the salicylate concentration, the higher the interference. Also, at a certain concentration of salicylate, the relative error in the albumin concentration increases with the level of albumin in the sample. However, the relative error and consequently the interference is negligible at salicylate concentrations in the therapeutic range (<2 × 10<sup>-3</sup> mol/L).

PRECISION

Within-day and day-to-day precision were studied for three serum pools with albumin concentrations at the low, middle and high region. For the day-to-day precision 10 aliquots of each pool were frozen and analyzed over a period of 16 days. No recalibration was made during this period. As shown in Table 3, the within-day and day-to-day coefficients of variation (CV's) were 1.1 to 1.5% and 1.6 to 2.3%, respectively.

CORRELATION WITH OTHER WIDELY APPLIED METHODS

We compared serum albumin concentrations as measured by the proposed PIC-method, with those obtained by the BCP-spec method and by the BCG method. Ninety-three specimens from normal individuals and patients were analyzed. Albumin concentrations were in the range of 12 to 52 g/L.

Figure 7 shows that the correlation between the PIC and the BCP-spec method is very good. The regression equation is  $y(\text{PIC}) = 0.98 x(\text{BCP}) + 0.76$  (in g/L) and the correlation coefficient,  $r = 0.979$ .

Results by the PIC method were lower than by the BCG method (Figure 8). The regression equation was  $y(\text{PIC}) = 0.95 x(\text{BCG}) - 1.3$  ( $r = 0.947$ ).

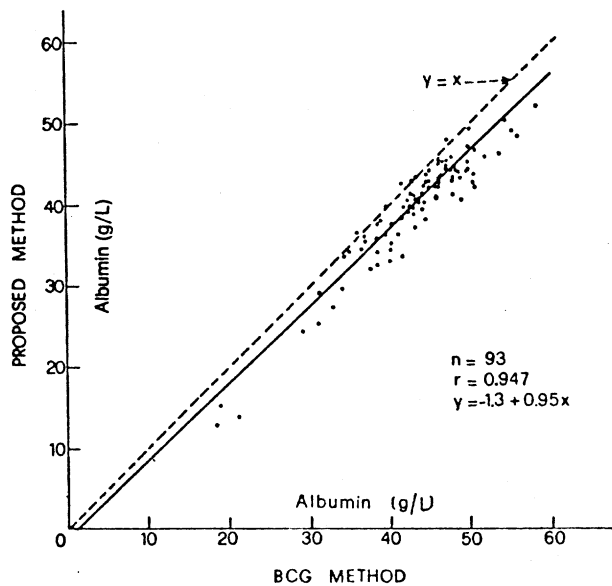


Figure 8 — Correlation between the proposed potentiometric assay (picrate as ligand) and the BCG spectrophotometric procedure. Broken line indicates the ideal situation where  $y = x$ .

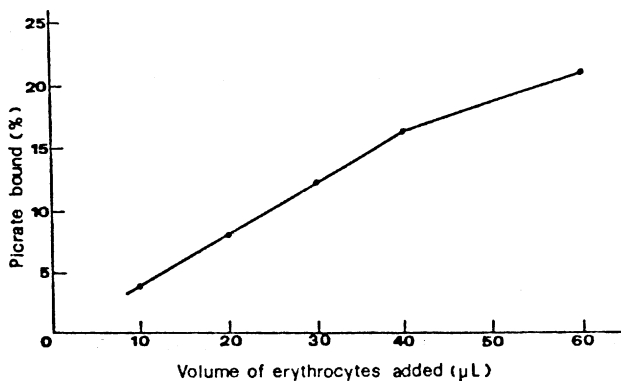


Figure 9 — Binding or entrapment of picrate by erythrocytes. Total picrate concentration is  $2.04 \times 10^{-4}$  mol/L. Isotonic phosphate buffer of pH 7.0.

### Assays in plasma and whole blood

#### INTERFERENCES

Hemoglobin at 4 g/L (erythrocyte hemolysate), fibrinogen (bovine) at 5 g/L and heparin at 450 U/mL do not interfere with the proposed method.

The effect of intact erythrocytes deserves special mention. The cells were isolated from plasma by washing three times with 0.154 mol/L NaCl and one time with the assay buffer. The last supernatant of this procedure contains essentially no albumin. Finally, the cells were centrifuged at  $2000 \times g$  for 15 min and the supernatant discarded. Initially, we applied the same procedure as for serum albumin assay except that solution 5 was used instead of solution 2. The apparent albumin concentration of a 50  $\mu$ L sample of packed red cells was 4 g/L. When we increased the picrate concentration in solution 5 to  $2.04 \times 10^{-4}$  mol/L the same sample of packed cells gave an apparent albumin concentration of 17 g/L. We conclude that the erythrocytes bind or entrap picrate anions.

TABLE 4  
Albumin Determinations in Plasma and Whole Blood of 12 Individuals

Sample	Hct*	Albumin (g/L)		
		In plasma Measured	In whole blood Expected	In whole blood Measured
1	40	33.8	20.3	20.9
2	42	42.5	24.7	25.4
3	29	35.8	25.3	25.4
4	40	39.9	24.1	23.0
5	43	40.2	23.1	23.1
6	32	36.8	25.1	24.6
7	41	46.1	27.1	27.2
8	46	50.2	27.1	26.8
9	41	46.1	27.0	27.8
10	48	49.6	25.8	25.8
11	43	47.2	26.7	24.6
12	44	50.4	28.2	28.2

\*Hct = hematocrit.

In Figure 9 the percentage of picrate anions bound as a function of the volume (in  $\mu$ L) of packed cells added into a 2 mL aliquot of  $2.04 \times 10^{-4}$  mol/L picrate solution (isotonic, pH 7.0), is shown. The free picrate concentration after each addition was calculated from the difference in EMF before and after the addition of the cells and from the electrode slope. As shown in Figure 9, about 20% of total picrate present is bound as a consequence of the addition of 50  $\mu$ L of erythrocytes.

When we increased the ionic strength of solution 2 by adding NaCl to a concentration of 1 mol/L, we observed that the interference remained the same (a 50  $\mu$ L sample of erythrocytes gave an apparent albumin concentration of about 4 g/L) and the sensitivity of the method decreased (calibration curve with a lower slope). Finally, we succeeded in eliminating the interference of erythrocytes by increasing the pH of the assay from 7.0 to 8.0 (see Procedures) using solution 4. A 50  $\mu$ L sample of packed cells gave an apparent albumin concentration lower than 0.5 g/L. The percentage of picrate bound to erythrocytes at this pH was lower than 3%.

#### CORRELATION BETWEEN RESULTS OF PLASMA AND WHOLE BLOOD

Albumin was quantified in plasma and whole blood of 12 individuals by the proposed method. From the values obtained for each plasma sample and the hematocrit, we calculated (applying Eq. 14) the expected albumin concentrations in the blood of each individual. These values are in close agreement with those obtained by direct analysis of blood samples. The results are presented in Table 4.

#### PRECISION

We assessed within-day precision by replicate analysis of a blood sample containing 28.2 g albumin/L. The CV was 1.7% ( $n = 10$ ).

## Discussion

The present method quantitates the binder by using a specific anionic ligand. The ligand-binder interaction is monitored potentiometrically because we wanted to take advantage of the property of ISE to monitor the free ligand activity (concentration) in the presence of bound ligand. The fundamental difference between our method and those previously described for binder quantitation is that we keep the free, rather than the total, ligand concentration constant. Our theoretical model was derived directly from the generalized theory of binding. According to this theory, the concentration of bound ligand is proportional to the total binder concentration only if the free ligand concentration is constant. The final equation (Equation 13) which forms the basis of the calibration curve is exact; that is, no approximations were introduced for its derivation and it is valid for any number of binding sites.

With the proposed procedures, albumin can be quantified in serum, plasma and whole blood. We tested BCP and picrate as ligands. The picrate ISE has already been described in the literature (19). The BCP electrode is new and it will be described elsewhere. With BCP as ligand, the linearity of the method extends to at least 160 g/L, and compares advantageously with the respective spectrophotometric procedure which exhibits linearity up to 60 g/L (13) (Figure 2). The wide linear range that is unaffected by pH (Figure 3) is predicted theoretically by Equation 3.

Figures 2 and 4 show that if the free ligand concentration is decreased, the slope of the calibration curve increases. This behavior is explained by examining the factor

$$A = \frac{FV_s}{C_f} = \sum_{i=1}^m \frac{n_i k_i}{1 + k_i C_f} \cdot V_s$$

which is the slope of the calibration curve and increases with decreasing  $C_f$  (and vice versa). Furthermore, the lower the  $C_f$ , the greater the potential shift ( $\Delta E = E_2 - E_1$ , Figure 1) after sample addition. For example,  $\Delta E$  values were 2.7, 4.5 and 9.0 mV for  $C_f = 3.26 \times 10^{-4}$ ,  $8.00 \times 10^{-5}$  and  $3.26 \times 10^{-5}$  mol/L, respectively, after adding 50  $\mu$ L of a 8.9 g/L HSA standard (Figure 4). Another reason to select low  $C_f$  for the assay is that the selectivity improves because there is little ligand available to react with interfering substances. With picrate as ligand we chose a  $C_f$  value of  $3.26 \times 10^{-5}$  mol/L as optimum. At concentrations below  $10^{-5}$  mol/L, the electrode response is not satisfactory.

Changes in pH have no effect on the linearity, but they affect the slope of the calibration curves (Figures 3 and 5). This is obviously due to changes in the binding parameters. It is generally accepted that by decreasing pH, the binding sites of albumin as well as the intrinsic binding constants for anions increase (1). This is expected, since at low pH the dissociation of the carboxylic groups of the albumin molecule decreases and also, basic groups (e.g. amino-groups) become protonated. These combined effects favor the attraction of anionic species to the binder. Moreover, the transition of albumin from the "N" (Normal) to the "F" (Faster migrating) form occurring in the pH region of 2–4 (1)

results in the unfolding of the molecule and the appearance of hidden binding sites. The increase of the binding sites and of binding constants make the factor  $F$  of Equation 3 greater and the calibration curve steeper. The phenomenon is more dramatic with BCP as ligand.

The effect of pH on the binding of BCP to albumin is difficult to assess by spectrophotometry, since at low pH the binding does not result in any changes in the absorbance of the solution. A detailed potentiometric study of the binding of BCP and BCG to albumin will be published elsewhere.

With picrate as ligand, the slope of the calibration curve is greater at pH 4.0 (acetate) than at pH 2.5 (citrate). We attribute this to competition of citrate with picrate for the same binding sites on the albumin molecule.

It is imperative, in the proposed method, that the binding of the ligand, as well as the response of the sensor used, is fast. Otherwise, during the addition of the ligand, to adjust the free concentration to its initial value, negative or positive errors are observed, respectively. We encountered the problem of surpassing the correct volume  $V_c$ , with BCP as ligand, in experiments with pH values  $<4.0$ . The binding of picrate and the picrate electrode response at all pH values tested were instantaneous. The same is also true for BCP but at pH  $>4.0$ .

For the calculations, we do not use the values of the electrode parameters,  $E_0$  and  $S$ . However, we assume that they are constant during the assay period. This is reasonable since one assay lasts 2–3 min. We also assume that the sample addition does not cause any change to the liquid junction potential (see below).

In the albumin assay, if the ligand binds to other serum components, positive errors appear. Negative errors can be caused by substances which displace the ligand from its binding sites. With BCP as ligand, the sensitivity of the assay is better than that with picrate, since we have a 200-fold sample dilution compared to 40-fold for picrate. Nevertheless, we chose picrate as the appropriate ligand because of its specificity. The potentiometric BCP assay exhibits positive bias (Table 2) even at pH 5.2 and with the assay conditions of Pinnel and Northam (13). We attribute this bias, which interestingly enough does not exist in the spectrophotometric version of the BCP assay, to the binding of BCP by other serum constituents.

Negative errors can be caused by salicylate, that are significant only at concentrations greater than the upper therapeutic level (Figure 6). Probably, the errors are due to displacement of picrate by the drug. The correlation between the proposed method and the widely accepted (13, 21–25) BCP spectrophotometric method is very good (Figure 7). The BCG method (30 s reading) still gives higher values (Figure 8) for reasons discussed thoroughly in the literature (10, 11, 26–28).

The binding of picrate to albumin was first reported by Teresi and Luck (29) and it has been used to develop a potentiometric continuous-flow assay for albumin in serum by Diamandis *et al.* (30). In that procedure, the total rather than free picrate concentration was kept constant. For the calibration curve, the potential shift



$\Delta E$  was used as the dependent variable. For the reasons described above, the linearity and sensitivity of that procedure were limited. The new procedure offers significant advantages over the original report.

The ability to measure analytes in whole blood is desirable especially for bed-side, doctors' office or home monitoring. A number of analytes are now measured in whole blood e.g. pH, blood gases and electrolytes (31–33) all by potentiometric sensors. When we applied the present method directly to whole blood, we encountered problems caused by the presence of erythrocytes in the measuring solution. The use of washed albumin-free packed erythrocytes as sample caused a potential shift and consequently, apparent albumin readings. We considered two possibilities: (a) that the erythrocytes change the liquid-junction potential, and (b) the erythrocytes bind or entrap picrate anions. Sigaard-Andersen *et al.* (32) found an increase in the liquid-junction potential of about 0.5 mV, when approximately 40% of the measuring volume is erythrocytes instead of water. In view of these findings, we concluded that the potential shift was not due to liquid-junction effects since in our system, only 3% or less of the measuring volume is erythrocytes whereas the potential shift was 4.5 mV (at pH 7.0). We attributed the potential shift to picrate binding or entrapment into the erythrocytes (Figure 9). This hypothesis is further supported by the observation that the shift was negligible at pH 8.0. This dramatic reduction is characteristic of the binding phenomenon and it is unlikely to happen if the shift is due to change in the liquid-junction potential.

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