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New Automated Dye-Binding Method for Serum Albumin Determination with Bromcresol Purple

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We describe a new automated dye-binding method for serum albumin determination with bromcresol purple (BCP) that has several advantages over an existing bromcresol green (BCG) method. The continuous-flow method is sensitive, linear, and precise, with negligible sample interaction at an analytical rate of 60 samples per hour. Unlike BCG, BCP did not react with an albumin-free serum globulin preparation or pure human transferrin solutions. Reaction with serum was instantaneous; in contrast, BCG exhibits a slow nonspecific reaction with some specimens. The specificity of BCP was demonstrated by good agreement with results of "rocket" immunoelectrophoresis (EIA) where y(BCP) = 0.95x (EIA) + 1.72. The BCG method overestimated serum albumin concentration where y(BCG) = 1.01x (EIA) + 6.77. Precipitation, which affects the BCG method, was not observed with BCP. Blank corrections were negligible, salicylate did not interfere, and bilirubin affected the method only if present in very high concentration. The method offers a solution to the poor accuracy of existing BCG methods while retaining many of their desirable features.

Additional Keyphrases: *electroimmunoassay, bromcresol green methods compared · specificity*

Dye-binding methods for serum albumin determination are simple, rapid, economical, and precise. Bromcresol green (BCG) is widely used and many methods have been described (*1–8*). BCG is less susceptible to interference than 2-(4′-hydroxyazobenzene) benzoic acid (*1*), and is sensitive, but has several disadvantages. The BCG/albumin complex may partly precipitate causing turbidity and the "negative baseline effect" (*1, 3, 9*). The absorptivity of the complex depends on the source of the albumin (*10*), so the choice of calibration material is critical. Most serious is the poor agreement between results by BCG methods and specific methods (*11–15*). Consequently, the IFCC Expert Panel on Proteins has recommended that BCG methods should be used only for screening purposes (*16*).

BCG overestimates serum albumin concentration because of a nonspecific reaction with some serum proteins. These proteins are found in the α_1-, α_2-, and β-globulin fractions and include the acute-phase reactants (*14, 17*). γ-Globulins do not react with BCG (*2, 17*). The nonspecific reaction is slow but the reaction with albumin is immediate, so that the error of the BCG method depends in part upon the duration of color development in the analytical system (*14*).

It was hypothesized (*18*) that increased reagent pH would reduce the number of weak electrostatic dye/protein interactions with a consequent reduction in the nonspecific reaction, as is the case with phenol red (*19*). However, direct colorimetric BCG methods are restricted to a pH near 4 because of the high reagent absorbance and decreasing effect of albumin at higher pH values. Consequently, we sought a new dye. Bromcresol purple (BCP) was investigated in detail for three reasons. This dye changes color from yellow to purple in the pH interval 5.2–6.8, permitting use of a reagent at about pH 5. BCP is structurally similar to BCG, so we hoped that the desirable properties of the latter would be retained. Louderback et al. have reported[2] that BCP reacts only with albumin, and a manual method for this dye has been described (*20*).

We have devised an automated method and compare here its performance with that of an established BCG method and a specific electroimmunoassay method (EIA), with encouraging results.

Materials and Methods

BCP Method

Stock BCP solution, 40 mmol of BCP per liter of absolute ethanol. Dissolve 0.54 g of BCP (pH Indicator Grade; B.D.H. Chemicals, Poole, England) in 15 ml of absolute ethanol. When a clear orange solution is obtained, dilute to 25 ml with absolute ethanol. The reagent is stable at 4 °C for at least three months. Preliminary examination indicates that BCP from other manufacturers (Koch-Light Laboratories Ltd., Colnbrook, England; Aldrich Chemical Co. Inc., Milwaukee,

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[2] Louderback, A., Mealy, E. H., and Taylor, N. A., A new dye binding technic using bromcresol purple for determination of albumin in serum. *Clin. Chem.* **14,** 793 (1968). Abstract.

Wis.; and Fisons Scientific Apparatus Ltd., Loughborough, England) perform similarly in their reaction with albumin.

Brij-35 solution. Dissolve 25 g of "Brij-35" (polyoxyethylene lauryl ether; Sigma Chemical Co., St. Louis, Mo.) in distilled water, with warming, and dilute to 100 ml.

Stock acetic acid solution. Dilute 150 ml of glacial acetic acid (AR grade) to 1 liter with distilled water.

Working BCP reagent, 40 μmol of BCP per liter. Dissolve 10 g of sodium acetate trihydrate (AR grade) in about 800 ml of distilled water. Add 10 ml of stock acetic acid solution, 1 ml of Brij-35 solution, and 1 ml of stock BCP solution, and dilute to 1 liter with distilled water. Check the pH and if necessary adjust to 5.2 ± 0.03 with stock acetic acid solution or 0.5 mol/liter sodium hydroxide solution. The reagent is stable for at least a week at room temperature.

Sodium chloride solution. Dissolve 9 g of sodium chloride in 1 liter of distilled water.

Standards. For this we used human albumin [cat. no. B5158; Dade Biochemicals, American Hospital Supply (U.K.) Ltd., Didcot, England] specified as 100% albumin by moving-boundary electrophoresis and assayed by the macro-Kjeldahl procedure. Reconstitute the contents of the vacuum-sealed ampoule in 3 ml of sodium chloride solution as recommended by the manufacturer and dilute with sodium chloride solution to give concentrations up to 60 g/liter.

Equipment. AutoAnalyzer I modules: Mk2 Sampler, Mk1 Pump, Colorimeter (with 15-mm **N** tubular flow cell), and Recorder (Technicon Instruments Co. Ltd., Basingstoke, England).

The continuous-flow system (Figure 1) gives a sample dilution of about 200-fold. The resampled stream is injected with air to minimize sample interaction (as used in a modified BCG method; E. C. Albutt, personal communication). Particular care is necessary to achieve a regular bubble pattern, or noisy peaks will result.

BCG Method

We used the method of Northam and Widdowson (*1*), with the standards described above, and measured absorbance at 636 nm. In this method, which is widely used in the U.K., a reagent with 40 μmol of BCG per liter and 1 ml of a Brij-35 solution (250 g/liter) in citrate buffer (50 mmol/liter, pH 3.8) is used.

Electroimmunoassay

The Laurell "rocket" technique was used. Serum and standards (as above) were diluted 250-fold with sodium chloride solution and 5 μl was applied to a well 2.5 mm in diameter, cut in a gel layer 1.5 mm thick. The gel contained 15 g of agarose per liter, monovalent anti-human albumin antiserum, and barbital buffer (50 mmol/liter, pH 8.6). After electrophoresis at a field strength of 10 V/cm for 3 h, the plates were pressed under filter paper layers, dried with a hair-dryer, and stained with Coomassie Brillant Blue R. Peak heights

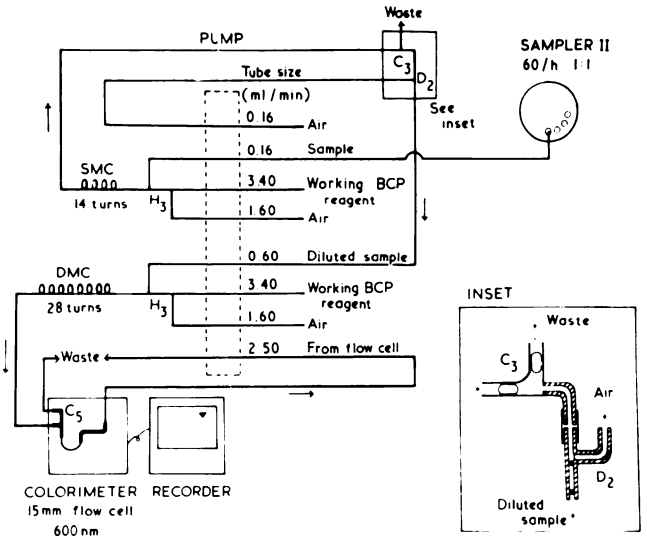

Fig. 1. Flow diagram for the BCP method
Inset shows air re-injection system

were measured, a standard curve was plotted, and unknown serum albumin concentrations were determined. Equipment and reagents were obtained from Hoechst Pharmaceuticals Ltd., Hounslow, England.

Results

Reaction between BCP and Albumin

Optimum conditions. The working BCP reagent (without Brij-35) exhibits absorbance maxima at 436 and 590 nm, with absorbances of 0.91 and 0.11, respectively. With increasing pH the absorbance at 590 nm increases, but that at 436 nm decreases. Addition of Brij-35 causes the opposite effect, a phenomenon also noted with BCG (*2*).

Addition of albumin to the working BCP reagent causes an increase in absorbance at 590 nm with a shift in the peak from 590 to 603 nm. The difference spectrum of albumin plus reagent vs. reagent shows an absorbance maximum at 603 nm.

We examined the effect of reagent conditions on absorbance of the albumin/BCP complex at 603 nm by adding 0.1 ml of a 40 g/liter solution of albumin to 20 ml of BCP reagent, using a reagent blank. Conditions other than those under examination were kept as in the original method (above). The absorbance increased gradually over the pH interval 5.1 to 5.6, from 0.275 to 0.324. Similarly, increasing the BCP concentration from 40 to 100 μmol/liter caused an increase in absorbance from 0.292 to 0.313. Addition of Brij-35 solution up to 0.5 ml/liter increased the absorbance, but from 0.5 to 1.5 ml/liter the absorbance was constant, declining gradually at higher Brij-35 concentrations.

Reagent conditions were chosen (*a*) to achieve the best compromise between maximum absorbance of the BCP/albumin complex and minimum reagent absorbance at 603 nm, and (*b*) to be such that small inaccuracies in the routine preparation of the BCP reagent (i.e., buffer, Brij-35, and BCP concentration) would have minimum effect upon the reproducibility of the method.

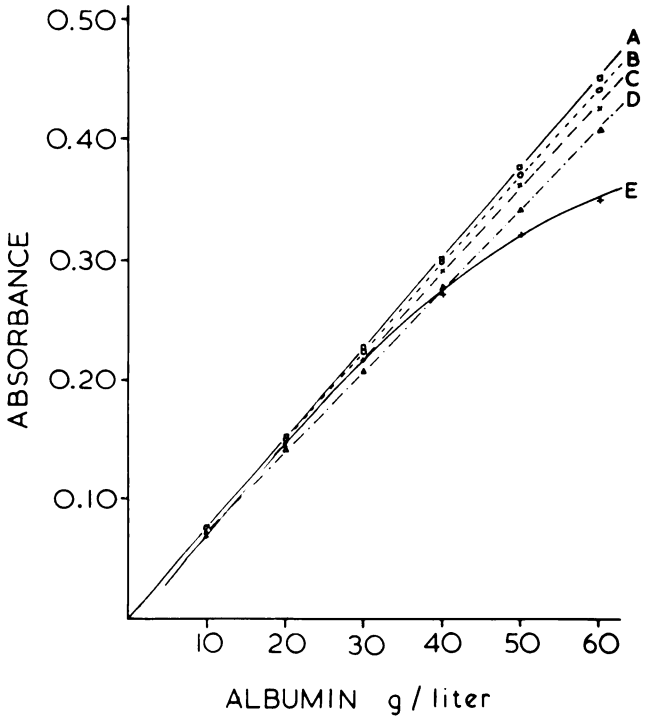

Fig. 2. Standard curves prepared with use of albumin from various sources *A*, purified human albumin (Dade Biochemicals); *B*, freeze-dried human serum (Q Pak I unassayed, Travenol Laboratories Ltd.); *C*, purified human albumin (Hoechst Pharmaceuticals Ltd.); *D*, purified human albumin (Sigma Chemical Co.). In *A–D*, absorbance was measured at 603 nm by the BCP method. *E*, purified human albumin (Hoechst Pharmaceuticals Ltd.); absorbance measured at 636 nm by the BCG method

Standards. Three purified human albumin preparations were obtained: that from Dade Biochemicals described above; from Hoechst Pharmaceuticals Ltd. (specified as >99% albumin, with no detectable protein inpurities); and from Sigma Chemical Co. (crystallized albumin containing 1–3 g of globulins per 100 g). These albumin preparations were dissolved in sodium chloride solution to give a concentration of 60 g/liter. We did not correct for moisture or other impurities. With the BCP method, concentration and absorbance were linearly related up to an albumin concentration of 60 g/liter (Figure 2). The Dade albumin preparation gave the highest absorbance with BCP (0.45 in a 1-cm light path at 603 nm). The Hoechst and Sigma preparations gave lower absorbances, possibly reflecting the presence of impurities and, in particular, the presence of moisture, because only the Dade albumin was supplied in vacuum-sealed ampoules.

A freeze-dried serum ("Q Pak I unassayed"; Travenol Laboratories Ltd., Thetford, Norfolk, England) was reconstituted in 6 ml of distilled water rather than the recommended 10 ml, to achieve the high albumin concentration, which was then electroimmunoassayed with use of a commercial assayed serum "standard" provided by Hoechst Pharmaceuticals Ltd. Linearity and absorptivity were similar to that given by the Dade albumin standard (Figure 2).

The linear range for the BCG method was narrower than in the BCP method and the maximum absorbance was less (Figure 2).

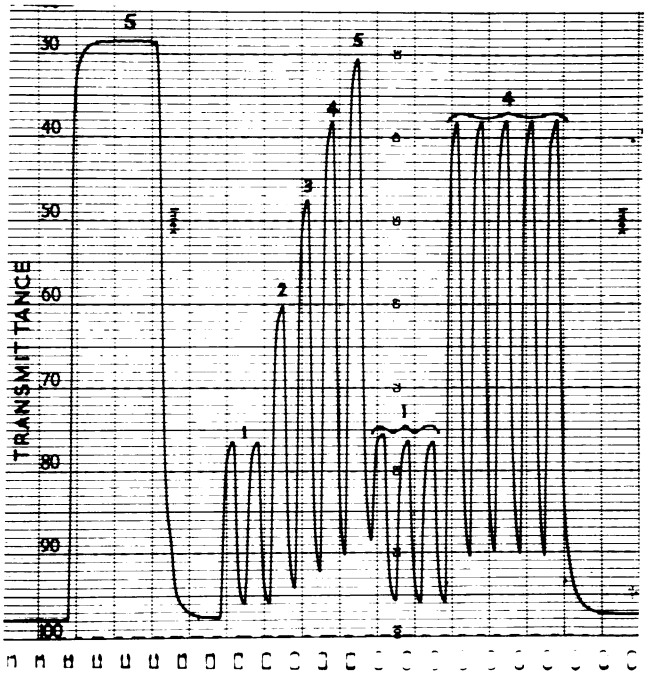

Fig. 3. Recorder trace of analysis of freeze-dried human serum

Q Pak I unassayed (Travenol Laboratories Ltd.) was reconstituted with distilled water to 57.5 g of albumin per liter. This was diluted in sodium chloride solution: *1*, 1 in 5; *2*, 2 in 5; *3*, 3 in 5; *4*, 4 in 5; *5*, undiluted. Chart speed, 50.8 cm/h

Sample interaction. To evaluate this, we analyzed groups of three specimens of low concentration, preceded by three of high concentration. At 60 samples/h, with a sample/wash ration of 1/1, serum containing 11.5 g of albumin per liter was increased to an apparent 12.1 g/liter when assayed next after serum containing 57.5 g of albumin per liter. Interaction from low to high specimens was negligible. Approach to the continuous aspiration plateau was within two transmission lines (i.e., 91% of steady state in terms of absorbance units, 97% in terms of transmission units) at a concentration of 57.5 g of albumin per liter (Figure 3).

Precision. Within-batch precision was measured by randomly distributing 20 specimens of albumin concentration 13.0 g/liter (serum pool diluted with sodium chloride solution) between 20 of concentration 35.9 g/liter (undiluted serum pool). For both concentrations the CV was 0.43%. To assess between-batch precision we prepared two serum pools with albumin concentrations of 23.1 g/liter and 39.6 g/liter (by pooling patients' sera with low and normal albumin concentrations, respectively), divided into aliquots, and stored at 4 °C. Aliquots of each serum pool were analyzed, in 20 batches each, on different days during four weeks. Each batch included eight to 15 serum specimens. We used the same manifold, stock BCP solution, stock acetic acid solution, and Brij-35 solution throughout this period.

Specificity. Albumin was selectively removed from serum by affinity chromatography on cyanogen bromide-activated agarose (Sepharose) gel covalently linked to Blue Dextran 2000 [Pharmacia Fine Chemicals, Pharmacia (Great Britain) Ltd., London] (*21*). The eluate from the gel columns was examined for the presence of albumin by immunoelectrophoresis against

Table 1. Apparent Albumin Concentration of Albumin-Free Serum Eluates Prepared by Affinity Chromatography

Nature of specimen	Total protein g/liter	"Albumin" concn, g/liter BCG method	BCP method	RID[a]
Crohns disease	36.0	11.0	0.5	0.7
Diffuse γ band on electrophoresis	31.0	7.0	0	0
Nephrotic syndrome	18.0	6.5	0	0
Glomerular nephritis	17.0	5.5	0	0

[a] Radial immunodiffusion.

monovalent antihuman-albumin serum and polyvalent antihuman serum (Hoechst Pharmaceuticals Ltd.). Comparison with the original serum showed that the precipitin arc corresponding to serum albumin had disappeared and all other detectable proteins remained. Eluates from the gel columns of serum from four patients were analyzed by the BCP method, the BCG method, and the Mancini radial immunodiffusion method (Hoechst Pharmaceuticals Ltd.) (Table 1). The apparent albumin concentrations, assayed by the BCG method, were between 23 and 32% of the total protein concentration of the eluate. Albumin was not detected by either the BCP or the radial immunodiffusion methods, except in specimen 1, where albumin was also detected by immunoelectrophoresis.

Human transferrin (Hoechst Pharmaceuticals Ltd.), specified as >99% pure with no detectable impurities, was dissolved in sodium chloride solution to give concentrations in the range 5 to 40 g/liter. We detected only one precipitin arc by immunoelectrophoresis against a polyvalent antiserum. Each solution was analyzed by the BCP and BCG methods. Transferrin had a significant reaction with BCG (Table 2) even in the presence of albumin but showed no reaction with BCP.

Color development of the BCP reagent with purified human albumin or serum was instantaneous (less than 10 s after mixing) and was stable for at least 1 h. No reaction was observed with the albumin-free serum preparations or transferrin solutions, either immediately or after 1 h. Some serum samples exhibited a slow reaction with BCG while with other sera and purified human albumin solutions color development was instantaneous. For transferrin solutions, color development with BCG continued over a period of 1 h (Table 3).

We estimated the albumin concentration of various animal-based serum specimens, using Dade human albumin for calibration. Two freeze-dried bovine-based quality-control sera (Wellcome Reagents Ltd., Beckenham, England) had apparent albumin concentrations of 31.0 and 36.0 g/liter by the BCG method, but only 12.6 and 12.9 g/liter, respectively, by the BCP method. Pure bovine serum albumin (Hoechst Pharmaceuticals Ltd.) in sodium chloride solution at a concentration of 40 g/liter gave an apparent albumin concentration by the BCG method of 43.0 g/liter, but only 15.0 g/liter by the BCP method. An unassayed equine serum had an albumin concentration of 39.0 g/liter by the BCG method, 23.9 g/liter by the BCP method.

Table 2. Reaction of BCG (*a*) with Pure Human Transferrin Alone, and (*b*) with Pure Human Transferrin in the Presence of Pure Human Albumin

Transferrin concn	(a) Apparent albumin concn	% of transferrin assayed as albumin
g/liter		
5.0	4.3	86
10.0	6.5	65
20.0	9.5	47
30.0	13.5	45
40.0	16.5	41

Transferrin 6.7 g/liter + pure human albumin	(b) Apparent albumin concn.	% of transferrin assayed as albumin
g/liter		
8.5	12.0	52
17.0	19.0	30
26.5	28.0	22
33.5	35.0	22
42.5	43.5	15

Table 3. Rate of Color Development of the Reaction of BCG with Albumin, Serum, and Protein Preparations[a]

Time after mixing	Human albumin	Serum 1	Serum 2	Serum 3	Human transferrin	Albumin-free globulin prepn
			A			
10 s	.310	.330	.281	.221	.060	.041
30 s	.300	.321	.282	.221	.080	.050
1 min	.300	.320	.280	.223	.108	.053
5 min	.301	.321	.280	.231	.150	.061
30 min	.305	.320	.282	.249	.190	.094
60 min	.307	.321	.282	.250	.202	.095

[a] Absorbance at 636 nm vs. reagent at intervals shown, timed from mixing 20 μl of protein solution with 4 ml of BCG reagent.

Effect of Interfering Substances on the BCP Method

Blank correction. Sera were analyzed with use of a BCP reagent from which bcp itself had been omitted. Normal sera had a negligible absorbance. Icteric sera with bilirubin concentrations up to 500 μmol/liter required blank corrections of between 0.5 and 1.5 g of apparent albumin per liter. Similar corrections were required for grossly hemolyzed sera (approximately 1 g of apparent albumin per liter at 10 g of hemoglobin per liter). Grossly lipemic sera required the greatest corrections, 3.5 g/liter being necessary in one extreme example where the serum looked like milk.

Salicylate. Sodium salicylate, added to serum and to pure human albumin standards, did not interfere with

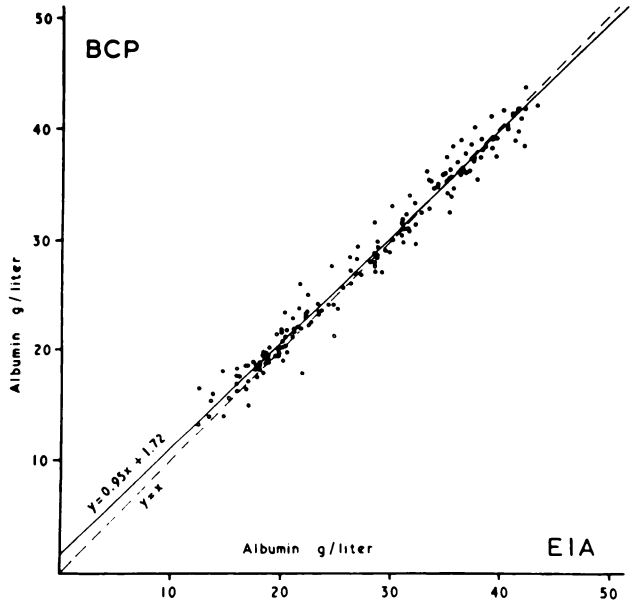

Fig. 4. Comparison of results by the BCP and electroimmunoassay methods

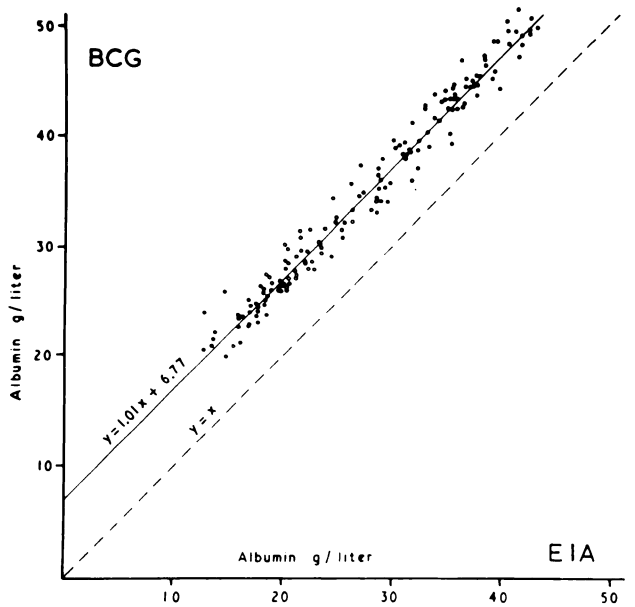

Fig. 5. Comparison of results by the BCG method and electroimmunoassay methods

the BCP method at concentrations up to at least 300 mg/liter.

Bilirubin. Purified bilirubin (Sigma Chemical Co., specified as essentially 100% bilirubin) was added to serum and to purified human albumin standards up to a concentration of 500 μmol/liter. Minor interference was noted at concentrations exceeding 170 μmol/liter. The apparent albumin concentration of a 40 g/liter solution was 39.4 g/liter and 38.3 g/liter in the presence of 250 μmol, and 500 μmol of bilirubin per liter, respectively. Sera with high bilirubin concentrations tend to be underestimated by the BCP method. Five sera with bilirubin concentrations between 113 and 192 μmol/liter were estimated by the BCP method and electroimmunoassay. Concentrations by the BCP method were lower by 0.2 g to 4.6 g/liter (mean difference, 2.4 g/liter). However, for 17 sera with bilirubin concentrations in the range 25 to 79 μmol/liter (mean concentration, 52 μmol/liter), analyzed by both methods, the mean difference in albumin concentration was only 0.1 g/liter.

Comparison of the BCP Method and BCG Method with Electroimmunoassay

Sera with albumin concentrations in the range 13 to 45 g/liter were assayed by the two dye-binding methods and by electroimmunoassay. Each analysis was performed in the same working day, to avoid sample deterioration. The same purified human albumin standards (Dade Biochemicals) were used for all three methods. Sera were selected to provide the maximum range of albumin, bilirubin, and globulin concentration. Sera showing other abnormalities (lipemia, hemolysis, presence of drugs, etc.) were also selected.

Results by the BCP and electroimmunoassay methods (Figure 4) agreed well, with the regression equation being $y(\text{BCP}) = 0.95x + 1.72$ (in g albumin/liter, n = 160, $r = 0.986$). Results by the BCG method were considerably higher than by electroimmunoassay (Figure 5), the regression equation being $y(\text{BCG}) = 1.01x + 6.77$ (n = 160, $r = 0.986$).

We further compared (with different sera to those used above) the BCG and BCP methods, with purified human albumin (Hoechst Pharmaceuticals Ltd.) for standardization. Similar results were obtained (Table 4).

A freeze-dried serum ("Validate"; General Diagnostics Ltd.) was standardized by electroimmunoassay with use of purified human albumin (Hoechst Pharmaceuticals Ltd.) Although the freeze-dried serum for calibration gave closer agreement between results by the BCG and electroimmunoassay methods, it was still not as good as that between electroimmunoassay and BCP methods (Table 4).

Discussion

The BCP method provides an alternative to existing BCG methods. The continuous-flow system described allows a high rate of analysis with good precision and little sample interaction.

In the BCG method (*1*), BCG tends to form a green fibrillar precipitate with albumin, an effect that is greatest at the isoelectric point (pH 4.3) of the complex (*9*). Precipitation is inhibited, but not eliminated, by the surfactant Brij-35. Neither precipitation nor the associated "negative baseline effect" (*1*) were observed with BCP, even in the absence of Brij-35, possibly due to the higher reagent pH used in the BCP method.

Several comparisons have been made between BCG and specific methods (*11–15*). Although all agree that BCG methods overestimate serum albumin concentration, the magnitude of the inaccuracy varies. This may reflect several factors: reagent composition (*10*), calibration material, time allowed for color development, and the source of specimens. In our study, agreement with electroimmunoassay was much better in the normal range when freeze-dried serum was used

Table 4. Regression Equations Describing Comparisons between BCP, BCG, and Electroimmunoassay (EIA) Methods, with Various Albumin Preparations Used for Standardization

	Pure human albumin stds from Dade			Hoechst	Freeze-dried human serum	
y	BCP	BCG	BCG	BCG	BCG	BCP
x	EIA	EIA	BCP	BCP	EIA	EIA
No. specimens (n)	160	160	160	120	80	80
Mean *x* (g/liter)	27.7	27.7	27.9	29.4	31.0	31.0
Mean *y* (g/liter)	27.9	34.8	34.8	35.8	32.5	30.4
Slope	0.95	1.01	1.06	1.02	0.85	0.95
Intercept	1.72	6.77	5.27	5.81	6.22	1.21
Correl. coeff. (*r*)	0.986	0.986	0.989	0.977	0.988	0.988
SD about regression line (S_y)	1.37	1.48	1.30	1.82	1.00	1.13

rather than purified human albumin for calibration (Table 4). Possibly interfering proteins in the freeze-dried material partly compensate for the presence of these proteins in test sera.

The slow rate of color development of BCG with interfering proteins (*14*) has been used to improve the accuracy of BCG methods by prompt measurement after mixing serum with reagent (*14, 25, 26*). However, an error of 3 g of albumin per liter was still present (*25*). Pure human transferrin and albumin-free serum preparations developed color slowly, but still had a significant reaction within 10 s (Table 3). Consequently, this method would not seem to be a complete solution to the problem and places considerable constraint on the analytical system that can be used.

The principal advantage of BCP is its specificity for albumin, as demonstrated by the lack of reaction with pure human transferrin or with the albumin-free serum preparations (Table 1), which contained most other serum proteins in their original concentration, as assessed immunochemically (*21*). The apparent albumin concentration of these solutions estimated by the BCG method was sufficiently great to explain the difference between results by electroimmunoassay and the BCG method.

In the BCP method, equine and bovine serum albumin are not suitable for use in calibration or quality control because their reactivity is much less than that of human albumin. However, nonhuman material is clearly unsuitable for use with specific immunochemical methods, and its use with BCG methods can be misleading (*10*).

Dye-binding methods are susceptible to interference by competing molecules. The BCP method is unaffected by salicylate. Purified bilirubin has little effect but specimens containing endogenous bilirubin at high concentration were sometimes underestimated, but rarely by more than two standard deviations from the regression line of BCP against electroimmunoassay. It has been suggested (*12*) that the BCG method similarly underestimates icteric sera. Consequently, the effect of bilirubin is undesirable but it is acceptable. Clearly, other molecules, particularly drugs and their metabolites, could also interfere and this is probably reflected in the degree of scatter of results about the regression line.

The high specificity of BCP for human albumin, its instantaneous reaction with albumin, and its susceptibility to interference by bilirubin could well be manifestations of the restriction of BCP binding to relatively few high-affinity binding sites on serum albumin. BCG binds to numerous fragments of bovine serum albumin, although it also has a high affinity for the fragment that is the site for bilirubin binding (*22*). Human serum albumin is known to have two general classes of binding site for anionic molecules other than fatty acids (*23*). Binding at the high-affinity site is by a combination of electrostatic and hydrophobic forces, while binding at the low-affinity sites, which are numerous but vary in number, is principally electrostatic. BCG can bind to two classes of binding site on serum albumin (*24*) but all that is required for the spectral change is immobilization on a cationic support (*9*) such as detergent micelles, gel, or protein. We hypothesize that the slow reaction of BCG with interfering proteins was due to electrostatic binding to cationic sites, which could be reduced in number by increasing the pH of the reagent. Investigation of this hypothesis might permit further improvement of the BCP method or the design of dyes specifically for albumin determination. However, the BCP method at its present stage of development offers better performance in most respects than do the BCG methods.

Because of the limitations of existing BCG methods it has been suggested (*17*) that abnormal sera should be analyzed by a specific method. Such methods are usually slow and impracticable for dealing with large numbers of specimens. The immunochemical methods require a supply of high-titer monovalent antisera, and are subject to several technical problems (*15*). The BCP method, however, shows excellent agreement with electroimmunoassay, yet retains the simplicity and capacity of existing dye-binding methods, permitting inexpensive "same-day-day" analysis of many specimens.

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