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High-Sensitivity Dye Binding Assay for Albumin in Urine

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We developed a dye-binding method for albumin in urine based on bis (3′,3″-diiodo-4′4″-dihydroxy-5′5″-dinitrophenyl)-3,4,5,6-tetrabromosulfonphthalein (DIDNTB), a dye that has a higher chemical sensitivity and specificity for albumin when compared to two other commonly used dyes. We prepared urine dipsticks with DIDNTB and certain other compounds to prevent "nonspecific" binding to the dipstick matrix. The detection limit for albumin with DIDNTB as the dye is about 10 mg/L. The extent of dye binding to proteins and other compounds was studied using ultracentrifugation and a selectively permeable membrane that permitted the passage of free but not bound dye; we believe this method is superior to photometric titration. The affinity of the dyes for albumin was found to be pH dependent with stronger binding at pH 1.8 than at pH 7.0. At pH 1.8, DIDNTB had a *ca.*10-fold greater binding coefficient to albumin when compared to the widely used dyes, tetrabromophenol blue (CI 4430-25-5) or bromophenol blue (CI 115-39-9). We developed a system that minimized nonspecific binding by the dye through the use of polymethyl vinyl ethers and bis-(heptapropylene glycol) carbonate. DIDNTB showed a greater chemical specificity for albumin when compared to most other proteins. The new albumin dipsticks are resistant to many potential interferences at substantial concentrations, making the dipsticks suitable to screen for albuminuria. J. Clin. Lab. Anal. 13:180–187, 1999.

Key words: dipstick urinalysis; dye binding of proteins; dry reagent analysis; strip testing; microalbuminuria; proteinuria

INTRODUCTION

Dye binding by proteins such as human serum albumin (HSA), Bence-Jones proteins (BJP), and gamma globulin (IgG) can be estimated by spectrophotometric titration. In this process, a protein solution is added to the dye with surveillance at one or more wavelengths to assess binding and to estimate the equilibrium-binding constant. The difficulties with this technique are the changes in absorptivity of the dye as protein is added, which causes a shift in the absorbence spectrum (1,2). Binding constants for proteins with dyes determined with the ultracentrifugation method that uses semipermeable membranes are more accurate (3).

We were interested in developing a urine dipstick that contained a dye with high affinity for albumin in order to make the dipstick more specific for albumin and to have much less affinity for other proteins such as IgG, Bence-Jones proteins, etc. Albuminuria is a harbinger of renal failure, and the correct identification of patients is important. Dipsticks are suitable for screening and can be used to detect albuminuria (4). The spectral properties of dyes we used are highly dependent on solvent polarity and pH; protein-bound dyes may also precipitate at acid conditions (5–9).

Albumin can be determined in a variety of ways. The simplest is dye binding in which the color change of the dye as it binds protein is observed. Many dyes have been used: 2-(4′-hydroxyazobenzene) benzoic acid (HABA), bromocresol green, bromocresol blue, bromophenol blue (BPB), and tetrabromophenol blue (TBPB). Dyes usually bind to the strong and weak binding sites of albumin; the strong binding sites are primarily responsible for the color shift of the dye. Of the many common proteins found in normal or abnormal urine, albumin gives the most color per gram of protein as it binds to the dye. A limitation of dye binding is that other proteins may also give a color change and thus falsely increase the albumin result. Also, most dye-binding methods do not have adequate chemical sensitivity to detect urine albumin concentrations of < 300 mg/L; a highly sensitive immunonephelometry is required. An albumin value of > 30 mg/L in urine is a harbinger of renal failure (10).

We describe here a dye, bis(3′3″-diiodo-4′4″-dihydroxy-5′5″-dinitrophenyl)-3,4,5,6-tetrabromosulfonphthalein (DIDNTB), that has sufficient sensitivity to detect urine albumin at ≥ 10 mg/L; it is much less affected by other pro-

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teins. Earlier work showed that a dipstick pad impregnated with DIDNTB had high chemical sensitivity and specificity for albumin and was suitable for detecting microconcentrations of albumin in urine. Microalbuminuria is usually defined as the spilling of > 20 mg/24 h (11). A simple dipstick test with a detection limit of about 10 mg/L should facilitate the identification of patients with trace concentrations of albumin in urine.

We had several goals: (1) to determine the binding constants for strong and weak binding of DIDNTB and TBPB to albumin and other proteins; (2) to test for competitive binding of possible interferents such as drugs, metabolites, polymers, certain proteins, contaminants, and the reagentless dipstick pads; and (3) to determine the effects of polyoxy polymers on dye binding. We wanted to estimate the concentrations at which other proteins start acting like albumin in binding to DIDNTB and TBPB.

MATERIAL AND METHODS

Equipment

Absorbence measurements were made in a Gilford (Oberlin, OH) Response II spectrophotometer. We used a Beckman Instruments (Brea, CA) model J2-21 ultracentrifuge; Centricon 3 filters with a 3,000 D Mr cutoff were from Amicon (Danvers, MA). All centrifugation was performed at 9,000 RPM; that gave a relative centrifugal force of 9,800*g*.

Reagents

We used DIDNTB and TBPB as dyes to bind albumin. Some limited studies were also carried out on bromophenol blue (BPB). Human serum albumin (HSA), fraction IV, and IgG fraction II were from Pentex (Kankakee, IL). Kappa monoclonal light chains, i.e., Bence-Jones proteins (BJP),were from Kallestad Laboratories (Austin, TX). All other reagents were ACS grade or better.

Two stock solutions of DIDNTB and TBPB, each 1.0 mmol/L, were prepared by dissolving 109.7 mg DIDNTB or 98.5 mg of TBPB in 100 mL of water containing 10 µL of 10 mol/L NaOH; alternately, they were dissolved in 100 mL of acetonitrile. Aqueous dilutions of the dyes were prepared to give concentrations in the range of 3.3×10^{-9} to 3.3×10^{-4} mol/L. We used buffers that were 50 mmol/L in both citrate and phosphate, and adjusted the pH as needed with 1 mol/L NaOH or 1 mol/L HCl. Stock solutions of 1.0 mmol/L (64.2 g/L) albumin, BJP (198.0 g/L), and IgG (26.0 g/L) were prepared in the above buffers. A particular buffer contained only one protein.

Reaction Conditions

We prepared solutions by combining 0.9 mL buffer, 0.1 mL of a protein solution and 0.5 mL of DIDNTB or TBPB in water or acetonitrile. We used final dye concentrations ranging from 3.3×10^{-8} to 3.3×10^{-4} mol/L, and final protein concentrations of 2.5×10^{-5} mol/L. We measured the dye binding by albumin, BJP, and IgG at pH 1.8 and 7.0 and at the dye concentrations stated above. All experiments were performed in duplicate with all three proteins for each dye at nine dye concentrations and pH values of 1.8 and 7.0 for a total of 216 experiments. The solutions were incubated at 25°C for 5 min before placing them in the Centricon ultracentrifugation tubes and spinning at 9,000 rpm for 10 min. Two hundred-µL portions of the filtrate were added to 1.5 mL of a 500-mmol/L potassium phosphate buffer, pH 1.8. The absorbance of both DIDNTB and TBPB were measured at 610 and 750 nm; the latter served as a nonspecific absorbance blank; the net absorbance was $A_{610}–A_{750}$.

Dye Binding to Protein

The fraction of dye passing through the membrane in the ultracentrifuge was determined by the concentration of dye in the filtrate at each concentration of dye, protein, and pH. We determined the mols dye bound per mol protein (r) from the absorptivities of the dyes, the mols of free dye in the filtrate, the mols of dye used, and the mols of protein present, i.e., r = [(mols total dye – mols free dye)/mols protein]. This assumes that the dye binds only to the protein and not to the container or filter membrane.

Dye Binding to the Membrane

During the high-speed centrifugation, essentially all of the solution passed through the membrane. The binding of dye to the membrane was < 1% in the absence of protein. We found that the reaction of DIDNTB or TBPB with the three proteins was essentially complete in < 1 min; we allowed a 5-min incubation time prior to centrifugation.

Preparation of Dipsticks Impregnated With DIDNTB or TBPB

The albumin dipsticks were produced in two separate soakings of the glass/cellulose filter paper (grade GF/CM30 obtained from Whatman International Ltd, Springfield Mill, Maidstone, Kent, UK). The first was with a solution that was 92% water and 8% ethanol, by volume, and contained a 250 mmol/L citrate buffer at pH 1.8 and the binding polymer, polyvinyl alcohol, at 2.5 g/L. The dipsticks were then allowed to dry at 110°C for 10 min. The second soaking solution contained 95% toluene and 5% tetrahydrofuran, by volume; the latter also contained 3.3 g/L dye, 1.4 g/L Lutanol (BASF, Ludwigshafen, Germany), and 3.75 g/L bis-(heptapropylene glycol) carbonate (KOK, Bayer AG, Leverkusen, Germany). The paper strips were dried at 110°C for 10 min for a second time, mounted on sheets of polystyrene, and cut into strips. After dipping the dried strips into various test and control solutions of proteins, analytes or potential

interferents, the reflectance spectra were measured with a Clinitek® reflectance photometer.

Competitive Binding Interferences

Solutions of potential interferents were prepared by combining 100 µL of a 1.0-mmol/L solution of the dye, 0.8 mL of 50 mmol/L citrate/phosphate buffer at pH 3.0, 100 µL of a 1-mmol/L solution of a possible competitive inhibitor in acetonitrile, and 500 µL of protein solution containing 1.0 g/L of either albumin or IgG. We also measured the absorbance of a blank containing only the potential binding agent in the buffer.

RESULTS AND DISCUSSION

Spectral Shifts of DIDNTB and TBPB Caused by Proteins

The influence of albumin or IgG on the absorptivity and peak absorbence wavelength of the deprotonated DIDNTB or TBPB buffered at pH 7.0 was determined with equimolar concentrations of the protein and dye. Albumin caused a decrease in absorptivity of about 10% at the peak absorbence of the dyes, while IgG showed no significant effect. This effect was solely dependent on the albumin concentration. We found the lowest absorptivities for the dye-bound albumin at 610 nm. A shift of up to 10 nm of the wavelength of the absorption maximum also occurred probably owing to intramolecular dye stacking (3).

Our results also showed that DIDNTB gave the most color change per gram of albumin at a pH of 1.5 to 2.5; the common dyes like TBPB and BPB gave an optimal response at a pH of 2.5 to 3.5. The lower pH used with DIDNTB is an advantage because it minimizes the interferences from some metabolites, preservatives, and drugs. DIDNTB is also more lipophylic than TBPB or BPB.

High- and Low-Affinity Binding of Albumin

A structure/reactivity study identified a substituted sulfonphthalein dye with a 10-fold increase in protein binding (12). DIDNTB had the greatest chemical sensitivity for albumin as compared to TBPB and BPB (see Fig. 1). Dye binding to albumin was affected by pH (see Table 1). At a pH of 1.8, high-affinity binding was observed, suggesting protonation or protein unfolding of internal sites. At pH 7, only low-affinity binding was observed. Dye binding to proteins like BJP and IgG was pH independent between pH 1.5 and 7.5. The magnitude and pH independence of these binding sites is similar to the low-affinity binding sites of albumin (1,13). Protein unfolding of internal sites or protonation is believed not to be involved in low-affinity binding.

The high-binding constant (K′) of albumin is thought to be owing to specific internal binding sites of the protein (14). The weaker interaction (K″) between the dyes and BJP or IgG is thought to be the result of "nonspecific" binding to the external protein sites. Both high-affinity (primary) and low-affinity (secondary) site-binding constants were determined for albumin by Scatchard analysis. The binding constants of albumin are about two orders of magnitude greater than those for BJP and IgG (see Table 1).

Dye binding to albumin was inhibited by increasing the concentration of the buffer, a finding that is consistent with the inhibitory role of the anions that are part of the structure of carboxylic acids (14). This type of inhibition is due to the competition between anions and dye for protein sites. To study competitive inhibition, dye binding to proteins was measured after equilibration of albumin with known compounds that bind to albumin (14). Published high-affinity constants and binding regions for these compounds measured at pH 7.0 are shown in Table 2 (14). Also shown is the reduced uptake of TPBP by albumin in the presence of competing binding agents. Dye binding to albumin was partially inhibited by all anionic competitive agents that we tested, e.g., palmitate. Nonionic compounds like phenylbutazone had no effect. Bilirubin and 8-anilino-1-naphthalenesulfonic acid (ANS) did not completely inhibit dye binding as would be expected by their published binding constants. It is likely that the change in pH

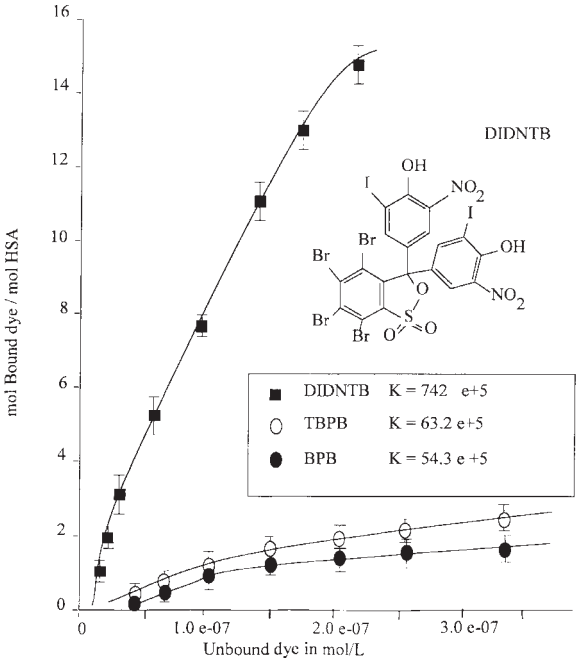

Fig. 1. Mols of bound dye per mol of protein (r) for DIDNTB, TBPB, and BPB. vs. concentration of unbound dye. The unbound dye was isolated by ultracentrifugation through a membrane as is described in the test. The dyes were present at the indicated concentrations in a solution that was 66.7% water-33.3% acetonitrile by volume. The data are for albumin at pH 1.8 in a buffer containing both 50 mmol/L phosphate and 50 mmol/L citrate. The protein concentration was 2.5×10^{-5} mol/L for all experiments, and the dye concentrations for all three dyes varied from 3.3×10^{-8} to 3.3×10^{-6} mol/L. Error bars represent one standard deviation.

TABLE 1. Strong and weak binding by albumin, BJP, and IgG

		DDNTB			TBPB		
		pH 1.8		pH 7.0	pH 1.8		pH 7.0
Solvent	Protein	K′ × 10^4	K″ × 10^4	K′ × 10^4	K′ × 10^4	K″ × 10^4	K′ × 10^4
33% acetonitrile[a]	HSAS	742	17.1	14.8	63.2	1.6	1.2
	BJP	16.2	n.m.	9.2	1.7	n.m.	0.8
	IgG	ppt.	ppt.	10.6	ppt.	ppt.	0.9
100% water	HSA	insol.	insol.	insol.	60.2	8.1	5.8
	BJP	insol.	insol.	insol.	9.6	n.m.	4.7
	IgG	insol.	insol.	insol.	ppt.	ppt.	5.5

[a]Effect of pH and solvent composition on strong (K′) and weak (K″) binding constants of albumin, BJP, and IgG. The buffer was the same as described in Figure 1. The protein concentrations were 2.5×10^{-5} mol/L throughout, and the concentrations of DDINTB and TBPB were varied from 3.3×10^{-8} to 3.3×10^{-4} mol/L. In three cases, the absorbance for the protein-bound TBPB was too low to measure (n.m.). In six other cases, the IgG formed a precipitate (ppt.) and could not be analyzed. DIDNTB is soluble in dilute alkali but not at pH 1.8 or 7.0 (insol.).

from 7.0 to 3.0, required to measure TBPB binding, promotes the binding of the sulfonphthalein dye to albumin.

Low-affinity binding interactions were dependent on the nature of the solvent; high-affinity binding was not. Low-affinity binding increased with solvent polarity as the result of weaker hydrophobic interactions between dye and protein. The high-affinity binding of albumin to TBPB did not change markedly with solvent polarity, suggesting that any hydrophobic binding is internal (see Table 1).

Nonspecific Binding of Dye by Various Compounds

We found nonspecific binding of DIDNTB to most polyaminoacids and positively charged polymers. Similar components in urine such as protein fragments, peptides, and polyamines such as spermidine also bound to DIDNTB (see Table 3.)

Nonspecific binding increased with the positive charge and the molecular weight of the analyte. The use of polymethyl vinyl ether (Lutanol, BASF AG, Ludwigshafen, Germany) greatly reduced the nonspecific binding of polyaminoacids to the dipsticks.

The greater percent inhibition of IgG binding by anions such as palmitate and SDS as compared to albumin suggested that the specificity of the dye-binding method for albumin could be enhanced (see Table 2). Therefore dye binding to albumin, lysozyme, IgG, and other proteins found in urine were measured in the presence and absence of a series of anionic acids added to the dye. A series of alkyl sulfonic and carboxylic acids were compared; those showing any effects are listed in Table 4. The optimal inhibition was determined by concentration-gradient experiments using 0.2, 0.8, 1.5, 3.0, and 6.0 mmol/L solutions of the anionic acid or polymers at 0.05% to 2.00% (w/v). The results in Table 4 confirmed that charged compounds are extremely strong inhibitors of dye binding to proteins, and they do

TABLE 2. Competitive binding of TBPB by albumin in the presence of competing agents

	HSA		IgG		HSA Binding	
Competing agent	Absorbance[a,b]	% Inhibition[c]	Absorbance[a,b]	% Inhibition[c]	K × 10^{6}[d]	Region[e]
TBPB only	1.40	0	0.70	0	0.6	3
ANS	1.06	24	0.18	74	10.0	3,8
Bilirubin	1.08	23	0.36	49	14	3
Dicoumarol	0.99	29	0.37	47	2.2	Unknown
Indomethacin	1.12	20	0.29	58	0.3	6
L-thyroxine	1.15	18	0.20	71	1.6	2
Palmitate	1.13	19	0.13	81	60.0	1
Phenylbutazone	1.40	0	0.70	0	0.2	Unknown
Salicylic acid	1.13	19	0.50	28	0.2	6
SDS	1.20	14	0.20	72	1.0	8
Tolbutamide	1.25	11	0.53	24	0.2	Unknown

[a]Absorbance at 610 nm corrected for the absorbance of the competing agent alone. This was done for TBPB only when a competing agent was present.
[b]The final concentrations of TBPB and the inhibitor were 67 μmol/L. The medium was a pH 3.0 buffer containing both 50 mmol/L citrate and 50 mmol/L phosphate.
[c]Percent of binding to competitive binding agents. For ANS, for example, this is calculated as [(140–106)/140]* 100.
[d]The binding constant, K, is the binding equilibrium constant for the compound to albumin at pH 7.0. For example, the binding constant of ANS to albumin is 10×10^6.
[e]Region of albumin molecule believed to be binding site.

TABLE 3. Competitive binding of DIDNTB with and without Lutanol[a]

Compound (molecular weight)	Relative amount of dye bound to dipstick	
	No Lutanol	Lutanol
Albumin, 65 kDa	100%	100%
GLYGLYGLY, 223 Da	8%	0%
GLYGLYHIS, 300 Da	7%	0%
IgG, 150 kDa	31%	18%
L-Histidine, 155 Da	5%	0%
Poly-D-lysine, 1–4 kDa	18%	6%
Polyethylenimine, 0.8 kDa	15%	10%
Poly-glycine, 2–5 kDa	9%	4%
Poly-L-arginine, 5–15 kDa	36%	3%
Poly-L-histidine, 5–15 kDa	27%	7%
Poly-L-lysine, 1–4 kDa	17%	6%
Poly-ornithine, 3–15 kDa	12%	6%
Spermidine, 145 Ka	15%	5%
Urinary protein fragments < 10 kDa	10%	0%
Urinary protein fragments < 20 kDa	24%	11%
Urinary protein fragments < 40 kDa	43%	13%

[a]The relative binding of albumin to the dipstick with and without 0.14% Lutanol. The dipstick pads were impregnated with DIDNTB and the strips were dipped into solutions containing the compounds shown above. The latter were all at 2.5 µmol/L, and buffered at pH 6.5 with 50 mmol/L phosphate. The Clinitek reflectance photometer at 610 nm was used to estimate dye binding to the pads. Note that Lutanol inhibited binding of DIDNTB in the dipsticks to nearly all the compounds tested witih the exception of albumin.

TABLE 4. Inhibition of albumin and IgG binding by various compounds and polymers to dipsticks containing DIDNTB[a]

	% inhibition observed	
	IgG	HSA
No additive	0%	0%
Decane sulfonic acid 6 mmol/L	23%	66%
Decanoic acid 6 mmol/L	2%	7%
Decanol 6 mmol/L	8%	3%
Dodecyl sulfate 6 mmol/L (SDS)	58%	90%
Hexadecane sulfonic acid 6 mmol/L	0%	2%
Hexadeconoic acid 6 mmol/L (Palmitic acid)	1%	2%
Hexanesulfonic acid 6 mmol/L	3%	6%
Octanesulfonic acid 6 mmol/L	4%	10%
Poly(sodium-4-styrene sulfonate) 0.10%	38%	40%
Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) 0.10%	38%	39%
Poly(styrene/maleic anhydride) 0.10%	12%	13%
Poly(vinyl alcohol) 0.10%	5%	6%
Poly(vinyl acetate) 0.10%	4%	7%
Poly(vinyl stearate) 0.10%	42%	15%
Poly(vinylsulfonic acid) 0.10%	51%	51%

[a]Inhibition of albumin and IgG binding by dye-impregnated dipsticks owning to various proteins, polymers, and other compounds. The effect of competitive inhibitors on the albumin dipsticks was studied by adding the potential inhibitor at 6 mmol/L, or 1 g/L in the case of polymers, to the dipstick-impregnating solution that also contained a 50 mmol/L, pH 6.5 phosphate buffer. The results are compared to those obtained with the standard albumin dipsticks. Note the strong inhibition by 6 mmol/L dodecyl sulfate (SDS).

not increase the chemical specificity for albumin. Dye binding of albumin was inhibited just like other proteins found in urine, including IgG. Most charged polymers cannot be used at a concentration of > 1 g/L without inhibiting binding to albumin. It was also possible that the anionic cellulose fibers of the paper support were acting as a competitive inhibitor.

Nonspecific Binding of Dye by Paper

Nonspecific binding of TBPB was observed with the paper support causing a decrease in the apparent pKa—the ionization constant of the dye—from 5.3 in water to 3.7 in paper. The dyes are weak acids, and in paper, they behave as stronger acids. The use of hydrophobic poly-oxypolymers, in particular KOK, reduced nonspecific binding of the dye by the paper support of the dipsticks. Treating the paper with KOK polymer prevented any change in the apparent pKa from that in water (see Fig. 2).

Interference Testing of Dipsticks for Albumin

Earlier protein dipsticks contained TBPB as the dye, and they lacked KOK. False-positive results were obtained with

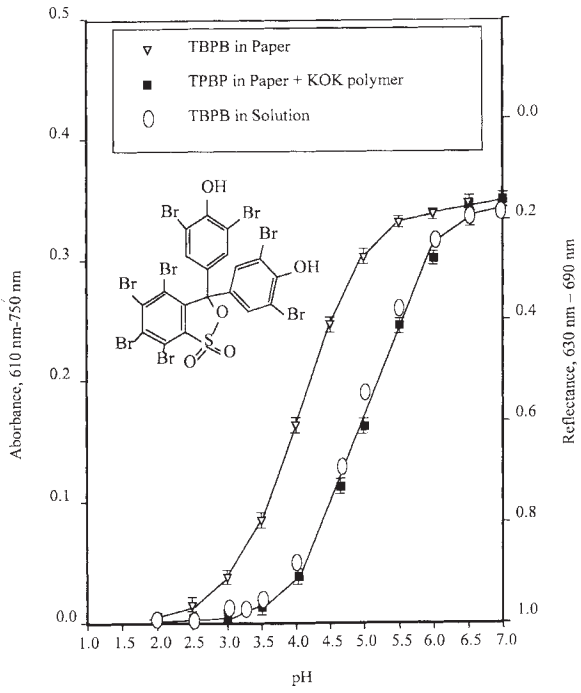

Fig. 2. Effect of KOK on dye binding to paper. Absorbence of 20 µmol/L TBPB in the absence of protein at pHs shown in buffer containing both 50 mmol/L phosphate and 50 mmol/L citrate ("TBPB in solution") and in 66.7% water and 33.3% acetonitrile by volume. Dipstick pads were saturated with 0.3 mmol/L TBPB in tetrahydrofuran and with KOK in one case. The reflectance was measured in the absence and presence of KOK polymer at 10 g/L. Note the elimination of nonspecific binding to paper by the KOK. Error bars represent one standard deviation.

highly buffered or alkaline urines particularly in the range of "trace" protein at about 150 mg/L and higher (15). In 1,367 urine specimens with an SG ≥ 1.030, 67% of the specimens gave a trace protein result. In some patients, protein concentration increased with specific gravity and trace was more likely in urines with a SG of ≥ 1.030. Using quantitative reference methods, 76% of randomly selected specimens from hospitalized patients with an SG ≥ 1.030 had positive protein values of > 150 mg/L while only 16% of those with an SG of < 1.005 had proteins of > 150 mg/L.

A buffered phosphate solution, as may be present in urine, shows the effect of buffer capacity on dye binding. The buffering capacity is the number of mols of strong base or acid required to change the pH by one unit. At a high buffering capacity of 66.5 mmol/pH unit, the color change of the strip is affected above a pH of about 8.0, even in the absence of

TABLE 5. Minimum concentrations needed to change the color of the DIDNTB dipsticks[a]

Metabolites	
Acetoacetic acid	2,500 mg/L
Ammonium chloride	2,060 mg/L
Ascorbic acid	4 g/L
Bilirubin	32 mg/L
Calcium chloride	800 mg/L
Citric Acid	650 mg/L
Creatine	100 mg/L
Creatinine	8 g/L
Formic acid	330 mg/L
Gentisic acid	800 mg/L
Glucose	400 g/L
Glycine	5.2 g/L
Glycolic acid	610 mg/L
Hemoglobin	25 mg/L
Imidazoleacetic acid	170 mg/L
Indoxyl sulfuric acid	6.2 g/L
Lactic acid	1.55 g/L
Oxalic acid	700 mg/L
p-Cresol	1,170 mg/L
Phenly acetic acid	620 mg/L
Potassium chloride	10 g/L
Sodium acetate, pH 5.5	8.2 g/L
Sodium bicarbonate, pH 8	10 g/L
Sodium chloride	20 g/L
Sodium dodecyl sulfate	50 mg/L
Sorbitol	36.4 g/L
Thymol	1 g/L
Tris	15.1 g/L
Triton X-100®	100 mg/L
Xylene	10 g/L
Drugs	
1-Phenyl-propanoic acid	2.4 g/L
Acepril (Captopril)	100 mg/L
Acetaminophen	8 g/L
Acetoazolamide (Glaucovox)	500 mg/L
Adalat (Nifedipine)	180 mg/L
Amoxicillin	10 g/L
Cardiazem (Diltiazem HC1)	360 mg/L
Cephalothin	5 g/L
Chlorpheniramine	200 mg/L
Ciprofloxacin	250 mg/L
Colistin	2.2 g/L
Diphenhydramine	50 mg/L
Demadex (Torsemide)	40 mg/L
Dextromethorphan	200 mg/L
Ephedrine	2 g/L
Erythromycin	1.1 g/L
Drugs (*continued*)	
Ethambutol	6 g/L
Sodium nitrate	30 mg/L
Sodium nitrite	30 mg/L
Sodium phosphate, pH 8	16.4 g/L
Urea	40 g/L
Uric acid	1.5 g/L
Urobilinogen	25 mg/L
Urine contaminates	
10 mol/L Sodium hydroxide	1 mL/L
6 mol/L Hydrochloric acid	5 mL/L
Ammonium sulfate	20 g/L
Benzalkonium chloride	10 mg/L
Boric acid	10 g/L
CHAPS	1 g/L
Chlorobutanol	10 g/L
Chloroform	10 g/L
Chlorohexidine	100 mg/L
EDTA	14 g/L
Ethanol	100 g/L
Glycerol	30 g/L
Guanidine	38 g/L
Microsoap®	10 mg/L
Octylglucoside	1 g/L
Sodium azide	2 g/L
Hydralazine	400 mg/L
Ibuprofen	4 g/L
Indomethacin	250 mg/L
Menthol	200 mg/L
Methyl-1-phenyl-1-propanol	2.4 g/L
Nitrofurantoin	250 mg/L
Norvasc (amlodipine besylate)	20 mg/L
o-Hydroxyhippuric acid	8 g/L
Penicillamine	10 g/L
Phenolphthalein	600 mg/L
Phenylbutazone	4 g/L
Pyridium (cetylpyridinium)	10 mg/L
Riboflavin	100 mg/L
Salicylic acid	1 g/L
Spironolactone	400 mg/L
Streptomycin	5 g/L
Sulfamethoxazole	200 mg/L
Sulfisoxazole	200 mg/L
Tetracycline	6 g/L
Theophylline	100 mg/L
Thiabendazole	700 mg/L
Tolbutamide	4 g/L
Tricarcillin	6.7 g/L

[a]Concentrations where interferences began for the DIDNTB-containing dipsticks. The specimens consisted of urine supplemented with the above compounds and with and without 30 mg/L albumin. A change in the Clinitek response of > 20% of the albumin response was considered to be a significant interference.

protein, and the strips will give inaccurate results (see Figure 3). At lower buffering capacities, the error is greatly reduced. We determined the buffering capacity of urine from > 5,000 hospitalized patients; the highest buffering capacity we ever observed was 35 mmol/pH unit.

With the polyoxypolymers in the dipsticks (e.g., KOK), the dipsticks were unaffected by changes in pH from 5 to about 8 when tested using contrived urine pools that had been supplemented with albumin. Also, there was no significant effect of the urine's pH with specimens from patients; the dipstick results were unaffected by pH in the range of 5 to 9.

The agreement of the albumin dipsticks containing DIDNTB with a quantitative immunonephelometric method for albumin (4) was not dependent on the urine's pH in the range of 5 to 8.2 or specific gravity (SG) in the range of 1.003 to 1.027. The pH and SG resistance of the strips were tested at pH 5.0, 6.5, 8.2, and at SGs of 1.003, 1016, and 1.027 in urine pools. All urine containing > 30 mg/L of albumin by the quantitative method gave a positive dipstick reading with no significant effect of a variable pH or SG.

Effect of Metabolites, Drugs, Contaminants, and Miscellaneous Compounds on Dipstick Results

Metabolites and drugs commonly found in urine generally did not interfere with dipstick measurements. An extensive list of metabolites, contaminants, drugs, and other compounds were examined for possible interferences with the DIDNTB dipsticks (see Table 5). In nearly all cases, the concentration of a possible interferent had to be extremely high before any effect on the dipsticks was found. Exceptions were Azo Gantrisin and Azo Gantanol, antimicrobials that contain dyes and color the urine. Our tests for interferences showed that contamination with detergents, soaps, antiseptics, or skin cleansers may give false-negative or false-positive dipstick results (see Table 5). The DIDNTB dipsticks were also affected by high concentrations of various urine preservatives. Boric acid at 1 g/L was an exception; it had no effect on the measurement of albumin by the new DIDNTB-impregnated dipsticks.

Effects of Other Proteins, Contaminants, and Preservatives on Dipstick Results

The dipsticks prepared with either TBPB or DIDNTB are not specific for albumin, and other proteins can give a positive result. Nevertheless, the dipsticks impregnated with DIDNTB are more selective for albumin than dipsticks impregnated with TBPB. Dipsticks impregnated with TBPB detected albumin and most proteins at a concentrations of > 200 mg/L, mucoprotein at > 600 mg/L, and globulin at >750 mg/L (see Table 6). The proteins we tested with the DIDNTB dipsticks had to be at much higher concentrations than albumin to produce the same amount of color per gram as does albumin. When comparing detection limits for TBPB and DIDNTB for albumin and Tamm-Horsfall protein, the TBPB sticks showed a 3-fold difference in the lower detection limit (200 vs. 600 mg/L) whereas the DIDNTB sticks showed a 25-fold difference (20 vs. 500 mg/L), i.e., DIDNTB was much more selective for albumin than was TBPB. Glycosyl albumin and beta-2-microglobulin gave the same amount of color per gram as did albumin.

TABLE 6. Effect of various proteins on protein and albumin tests[a]

	Detected by	
	TBPB Dipstick (mg/L)	DIDNTB Dipstick (mg/L)
Albumin	200	20
Glycosyl albumin	200	20
Beta-2-microglobulin	200	30
Transferrin	300	40
Myoglobin	200	50
Hemoglobin	200	50
Haptoglobin	300	50
Beta-2-glycoprotein	200	100
Retinol binding protein	400	200
Kappa light chains	400	200
Alpha-1-microglobulin	300	200
Alpha-1-antitrysin	300	200
Fc fragment of IgG	400	300
Lambda light chains	600	400
IgM	800	400
Tamm-Horsfall glycoprotein	600	500
IgA	1,000	700
Lysozyme	400	900
Prealbumin	700	900
IgG	1,000	900
Alpha-1-acid glycoprotein	1,500	1,500

[a]The specificity of the albumin dipsticks impregnated with DIDNTB were compared to the protein dipstick that used TBPB as the dye. The specimens were prepared from a urine pool that was free of all compounds with an Mr > 10 kDa. The minimum protein concentration needed to obtain a positive dipstick response is shown.

Storage Stability

Some urine specimens became unusable after 7 days at 25°C in closed containers, most likely owing to the presence of microorganisms. Storage at –50°C or 5°C preserved the albumin for at least 28 days. We do not recommend storage at room temperature.

CONCLUSIONS

DIDNTB is the best of the dyes that we tested for measuring urinary albumin. It has a much larger binding constant for albumin at pH 1.8 than does THPB. The interference from light-chain proteins and the normal immunoglobulins is much less for strips containing DIDNTB than for those containing TBPB. Anionic polymers interfere with dye binding to albumin, and they cannot be used to improve the discrimination

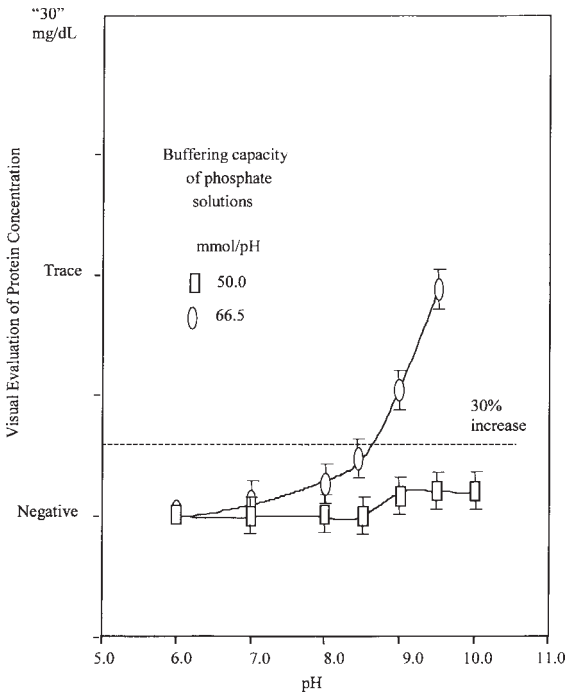

Fig. 3. Effect of buffering capacities on color change of dipsticks. Visual results of dipsticks after dipping into phosphate buffer at pHs shown with buffering capacities of 50 and 66.5 mmol/pH unit. Dipsticks were prepared by dipping into 3.0 mmol/L TBPB containing 1% KOK and 250 mmol/L citrate buffer. Error bars represent one standard deviation. Note that at a buffering capacity of > 50 mmol/pH unit, the buffer begins to change the color of the albumin pads on the dipsticks in the absence of proteins.

of DIDNTB for albumin. DIDNTB gives substantially more color per gram of albumin than all other proteins that are commonly found in urine; exceptions are glycosyl albumin and beta-2-microglobulin. With suitable buffering of the dipsticks, the shift in color of DIDNTB caused by albumin is not affected by urines with a buffering capacity of 50 mmol/pH or less, pH values between about 5 and 9, and SGs between about 1.000 and 1.030. KOK polymer and Lutanol were highly effective in reducing the nonspecific binding of dye to many potential interferents. With the exceptions of Azo Gantrisin and Azo Gantanol, the dipsticks are not affected by high concentrations of most metabolites, preservatives, contaminants, and drugs. DIDNTB is superior to TBPB and BPB for estimating albumin concentrations in urine. The dipsticks containing DIDNTB show more discrimination between albumin and other proteins than do dipsticks containing TBPB. The DIDNTB dipsticks could be used to screen for albuminuria. Dipsticks impregnated with DIDNTB have an increased chemical sensitivity and specificity as compared to TBPB.

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