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Removing Substances from Blood by Affinity Chromatography: *I. REMOVING BILIRUBIN AND OTHER ALBUMIN-BOUND SUBSTANCES FROM PLASMA AND BLOOD WITH ALBUMIN- CONJUGATED AGAROSE BEADS*

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Removing Substances from Blood by Affinity Chromatography

I. REMOVING BILIRUBIN AND OTHER ALBUMIN-BOUND SUBSTANCES FROM PLASMA AND BLOOD WITH ALBUMIN-CONJUGATED AGAROSE BEADS

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ABSTRACT Substances such as bilirubin that bind tightly to plasma proteins cannot readily be removed from blood. We describe here the use of affinity chromatography as a new approach to the removal of protein-bound metabolites and toxins from blood. Agarose beads were coupled via cyanogen bromide to human serum albumin so as to contain 30–50 mg of albumin/g wet wt. Such beads, when exposed to plasma from a patient with congenital nonhemolytic jaundice labeled with [¹⁴C]-bilirubin, bound more than 150 μg bilirubin/g of beads. The binding was saturable, concentration-dependent, relatively independent of flow rate, and reversible by elution with plasma, albumin, or 50% (vol/vol) ethanol. The beads could be repeatedly reused without loss of efficiency after ethanol elution and long storage in the cold. Salicylate, cortisol, and taurocholate, which bind weakly to albumin, were retarded by the beads but eluted with neutral buffer. Thyroxine, tauroolithocholate, chenodeoxycholate, and digitoxin bound tightly but were eluted with 50% ethanol. Digoxin did not bind at all. When whole blood was passed over agarose-albumin beads, bilirubin was removed, calcium and magnesium fell slightly, but red cells, white cells, platelets, clotting factors, and a variety of electrolytes and proteins were substantially unchanged. Agarose-albumin beads may be useful for removing protein-bound substances from the blood of patients with liver failure, intoxication with protein-bound drugs, or specific metabolic deficits. Furthermore, it may be possible to make useful adsorbents

by attaching other proteins to agarose or other polymer beads.

INTRODUCTION

Nonvolatile metabolic waste products and toxins are eliminated from the body predominantly by the kidney and liver. In general, low molecular weight water-soluble substances with little or no protein binding are filtered by the glomerulus, a process for which dialysis can provide an effective substitute when renal function is inadequate. In contrast, substances which are relatively insoluble in aqueous solution and/or which are transported in plasma tightly bound to plasma proteins are excreted predominantly by the liver. Such substances are not effectively dialyzable, even when the binding protein is in the dialysate (1). Although there are methods which may help the body to eliminate particular protein-bound substances such as bilirubin (2–8), no general method like dialysis exists.

Stimulated by the need to remove bilirubin (BR)¹ from a patient with the Crigler-Najjar syndrome (congenital absence of glucuronyl transferase in the liver) whose clinical condition was deteriorating, we have applied the principle of affinity chromatography (9) to the removal of BR and other albumin-bound substances from plasma and whole blood. We have coupled albumin to various polymer beads and passed plasma and whole blood over the beads in chromatographic columns. Albumin-conjugated agarose beads will remove albumin-

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¹ Abbreviations used in this paper: BR, bilirubin; HSA, human serum albumin; PBS, 0.14 M sodium chloride-0.01 M sodium phosphate, pH 7.2.

bound substances from plasma and blood, and they appear compatible with whole blood. They have been used subsequently in an extracorporeal hemoperfusion system in rats, as reported in the accompanying paper (10).

MATERIALS AND METHODS

Albumin. 25% human serum albumin (HSA) for injection from various manufacturers was supplied by the Bureau of Biologics of the Food and Drug Administration or was purchased from commercial sources. It was dialyzed in the cold for at least 18 h against 20 vol of the conjugation buffer, usually 0.1 M sodium bicarbonate, to remove sodium tryptophanate. The dialyzed albumin contained no detectable contaminating proteins when tested by immunoelectrophoresis with rabbit anti-whole human serum. Antibody raised in rabbits against one lot of the HSA reacted only with albumin in immunoelectrophoresis of whole human serum.

Agarose. Several kinds of agarose beads have been used, but in all the experiments reported here, Sepharose 6B 65-325 mesh (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and Bio-Gel A5m 100-200 mesh (Bio-Rad Laboratories, Richmond, Calif.) were used. In preliminary experiments, the albumin-binding capacities of Sepharose 2B, Sepharose 4B, Bio-Gel A1.5m, and A50m were found to be less than 6B and A5m. In recent experiments we have used Bio-Gel A5m because its larger bead size allows better flow of whole blood. In addition, there is more binding of bilirubin.

Buffers. Phosphate-buffered saline (PBS) (0.14 M sodium chloride-0.01 M sodium phosphate, pH 7.2) was supplied by the Media Unit of the National Institutes of Health. All other solutions were made from reagent grade chemicals and triple-distilled water. Ethanol solutions were made just before use by mixing absolute ethanol or 95% ethanol and triple-distilled water.

Radioactive materials. [^{14}C]BR was prepared as described earlier (11). For binding studies, trace quantities were added to jaundiced citrated human plasma obtained from a patient with the Crigler-Najjar syndrome. In general we used radioactivity rather than a chemical determination of BR (*vide infra*) because of the ease and accuracy with which a large number of samples could be processed without fear that BR degradation between the experiment and the assay would invalidate the results.

[^{14}C]salicylic acid (6.16 mCi/mmol) (New England Nuclear, Boston, Mass.) was recrystallized from boiling water with nonradioactive salicylic acid (Fisher Scientific Company, Pittsburgh, Pa.) to a final specific activity of 7.04 $\mu\text{Ci}/\text{mmol}$. [^{125}I]thyroxine was obtained from Abbott Laboratories (Chicago, Ill.). [^{14}C]taurocholate sodium (4.11 mCi/mmol), [^{14}C]taurocholic acid (1.75 mCi/mmol), and [^{14}C]chenodeoxycholic acid (7.82 mCi/mmol) were obtained from Mallinckrodt Chemical Works (St. Louis, Mo.). [^3H]digoxin (6.2 Ci/mmol) and [^3H]digitoxin (20 Ci/mmol) were obtained from New England Nuclear. All these substances were chromatographically pure as supplied by the manufacturers and were used without further purification. [^3H]cortisol was the gift of Dr. Lynn Loriaux. Chenodeoxycholic acid and both digitalis preparations were supplied in benzene-ethanol solutions. They were first diluted in ethanol, then further diluted in PBS before addition to serum.

In all experiments performed with these compounds,

the compound was added to serum and the mixture was incubated at 37°C for 15-30 min to allow equilibration before addition to the gel. Thyroxine, cortisol, and the bile salts were added in trace amounts. Salicylate was added in a therapeutic concentration, and the digitalis preparations were added in concentrations found in clinical digitalis toxicity (Table III). Indocyanine green (Hynson, Westcott, and Dunning, Inc., Baltimore, Md.) was added in a concentration of 12.5 mg/100 ml.

Preparation of agarose-albumin. After a series of experiments with various coupling methods and gels, we settled on a modification of the cyanogen bromide method of Axén, Porath, and Ernback to prepare agarose-albumin conjugates (12). 40 g of agarose was weighed while wet, then washed on a scintered glass funnel with distilled water. The gel was added with an equal weight of water to a TPX beaker (Nalge Co., Nalgene Labware Div., Rochester, N. Y.). A stirring bar, a thermometer, and a pH electrode were added and the beaker was placed in a hood on a magnetic stirring table. Cyanogen bromide (Eastman, Rochester, N. Y.), 10 g, was added and the pH brought to about 10.5 with 2.5 N sodium hydroxide. For the next 10 min, 2.5 N NaOH and small amounts of ice were added to the reaction mixture to keep the pH at 10-11.5 and the temperature at 18-22°C. Then a large quantity of ice was added, the activated gel was rapidly filtered and washed with at least 25 vol of ice cold 0.1 M sodium bicarbonate, and then added to a beaker containing HSA at 50-100 mg/ml in 0.1 M sodium bicarbonate. The beaker was rotated gently at 4°C overnight, and on the following day the gel was washed with PBS; 1.0 M NaCl-0.1 M sodium acetate, pH 5.0; 0.2 M borate saline, pH 8.4; PBS; and in recent preparations, 50% ethanol in water (vol/vol) followed by PBS. The washed gel was then filtered through a coarse nylon mesh tea strainer and defined by repeated suspension in PBS in a TPX cylinder and stored in a TPX container in the ice box. The amount of albumin bound was determined by measuring the absorbance at 280 nm of the first wash. No substantial amount of protein was removed by subsequent washes. The results obtained by using absorbance were in close agreement with those obtained using [^{125}I]HSA. Because a small amount of radioactivity leaching from the gels would have complicated the measurement of radioactive compounds, [^{125}I]HSA was not routinely used.

Usually, the agarose bound between 30 and 50 mg of HSA/g wet wt. This is equivalent to 30 to 50 mg HSA/ml (since the density of the wet gel is about equal to water), which is the same as the concentration of HSA in normal plasma.

Coupling at other pH's from 4 to 11 did not improve the albumin binding to agarose over that obtained in 0.1 M sodium bicarbonate. The prior conjugation of phloroglucinol to the beads with epichlorhydrin did not increase the amount of albumin bound (13). Acrylamide beads conjugated with hydrazine hydrate and coupled to albumin via the acyl azide (14) bound less albumin than cyanogen bromide-activated agarose.

Columns. Agarose beads were allowed to settle by gravity into disposable polypropylene columns with 8-mm internal diameter (Chromaflex, Kontes Glass Co., Vineland, N. J.). In experiments with serum or plasma, glass wool was used to retain the beads in the columns; in experiments with whole blood, nylon cloth (400 mesh) or stainless steel cloth (200 mesh) were fitted in the ends of the plastic columns between short lengths of Tygon tubing (Arthur H. Thomas Co., Philadelphia, Pa.).

Experimental design. Plasma containing the substance being tested was applied to an agarose-HSA column and the material that did not bind was collected along with PBS washes. We calculated the amount of substance bound as the difference between the amount added and the amount recovered with PBS washing. In most experiments, 1-5 ml plasma (or serum) equilibrated with radioactive compound was added to 3-10 g gel in a column and washed through with PBS. The flow was controlled with an LKB series 12000 pump (LKB Produkter, Bromma, Sweden) or by a needle on the column outflow and ranged from 0.3 to 3.0 ml/min. The unbound substances and the first wash with PBS were collected in 15-ml or 20-ml. Elution was then carried out with other salt solutions or ethanol in water and similar volumes were collected and aliquots taken for counting. Usually, two 15-ml fractions of PBS wash were collected and one 15-ml fraction of each eluate. Except as noted, there was rarely as much as 1 or 2% of the total unbound material in the second PBS fraction, and it was occasionally omitted for convenience. All experiments were performed at room temperature. Experiments with BR were carried out in a darkened room, and the columns and collecting vessels were jacketed in aluminum foil.

Determination of protein. Albumin concentrations were determined by measuring the absorbance at 280 nm in a Zeiss spectrophotometer PMII using an extinction coefficient of

$$E_{1\%}^{280} = 5.3 \text{ (15).}$$

Determination of radioactivity. Radioactivity in samples containing ^{14}C or ^3H was determined by adding up to 1 ml of aqueous sample to 10 ml of Aquasol (New England Nuclear) and counting for a suitable period in a liquid scintillation counter. All samples were then re-counted after the addition of ^{14}C - or ^3H toluene (New England Nuclear) as an internal standard. Duplicate vials were counted for all samples. The results are expressed as the average of the duplicate samples, each individually corrected for background and quenching. ^{125}I samples were counted in duplicate.

Bilirubin determination. The BR concentration in all plasma samples was determined by a slight modification of the van den Bergh method (16).³ Neither this method nor the method of Weber and Schalm (17), however, proved satisfactory when applied to the eluates containing ethanol but no protein. We determined that the van den Bergh method could be applied to samples containing from 5 to 70% ethanol as long as at least 0.5 g HSA/100 ml was present. A series of standard curves with 5-70% ethanol and 0.5-5.0 g HSA/100 ml were indistinguishable from one another. Therefore, the ethanol eluate was modified as follows: to six parts of 50% ethanol eluate were added, in order, 1 part of 0.1 N sodium hydroxide, 4 parts of HSA at 6 g/100 ml, and 1 part of 0.1 N hydrochloric acid. Thus the final solution contained 2 g HSA/100 ml

⁴ 4 ml of an appropriate dilution of plasma in saline was divided into two 2.0-ml portions. To one was added 1.0 ml of sulfanilic acid reagent (4 g of sulfanilic acid and 60 ml of concentrated hydrochloric acid made to 1,000 ml with water) and 1.5 ml methanol. To the other was added 1.0 ml of diazo reagent (0.3 ml of a 2% aqueous solution of sodium nitrite plus 10 ml of sulfanilic acid reagent) and 1.5 ml methanol. After 30 min, the optical density at 540 nm was determined.

and about 25% ethanol. Alkalinization of the eluate before the addition of HSA was found empirically to yield results which were both more reproducible and consistent with predicted values.

Studies on whole blood. Complete blood counts, leukocyte differential counts, and clotting and osmotic fragility studies were performed in duplicate on coded samples by the Hematology Laboratory of the Clinical Pathology Laboratory of the Clinical Center at the National Institutes of Health.

Blood chemistry studies were performed on coded samples by Bionetics Medical Laboratory (Falls Church, Va.).

Quantitative immunoglobulins were determined in duplicate on coded samples by using the ring diffusion method on Hyland Immunoplates (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.).

RESULTS

When plasma from a patient with congenital nonhemolytic jaundice with elevated levels of unconjugated bilirubin was passed over a column of agarose-HSA, a large proportion of the bilirubin bound to the beads and was not eluted by prolonged washing with PBS (Fig. 1). After the PBS washing, the column remained yellow. In order to establish that the coupled albumin, and not the agarose, was responsible for the BR binding (8), we passed equal quantities of jaundiced plasma over plain agarose, agarose conjugated with human gamma globulin, and agarose-HSA. As Table I demonstrates, only agarose-HSA bound any significant amount of bilirubin.

The amount of bilirubin bound to agarose-HSA was proportional to the initial concentration in the plasma. As expected, however, at any particular concentration there was apparent saturation of the gel when increasing volumes of plasma were presented (Fig. 2). Assuming

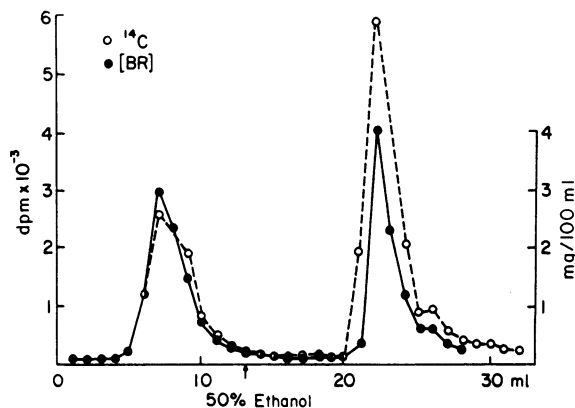


FIGURE 1 Binding of BR to agarose-HSA and elution by ethanol. 1 ml of plasma with 180 μg BR/ml labeled with ^{14}C BR was applied to a column with 3 g agarose-HSA and eluted with PBS followed by 50% (vol/vol) ethanol in water. BR was measured by the van den Bergh reaction and by radioactivity. The lower chemical values in the ethanol-eluted fraction are due to the degradation of BR before the chemical assay.

that one molecule of HSA has one strong binding site for bilirubin (18), one can calculate that agarose-HSA with 40 mg HSA/g of gel should strongly bind up to 300 μg of bilirubin/g. When exposed to plasma with an initial concentration of 24 mg BR/100 ml, some gels have bound more than 150 μg BR/g of gel, suggesting that the albumin lost less than half of its binding capacity as a result of the coupling.

The bound bilirubin could be completely eluted by either normal plasma or a solution of HSA at 40 mg/ml, but since the need to regenerate columns with plasma components would decrease their utility, we sought simpler means to remove the bilirubin. Neither 1 M sodium chloride nor 1 M sodium chloride-0.1 M sodium acetate, pH 5.0, removed any of the bound counts or color. Ethanol in water, 50% (vol/vol), however, removed all the bound material (Fig. 1). In 55 experiments in which the amount bound (amount added minus amount in the PBS washes) and the amount eluted by 50% ethanol could be compared, the ratio of eluted/bound was 0.99 ± 0.093 (SD).

After 50% ethanol elution, the beads were washed with PBS and stored in the ice box. Such used beads retained fully their capacity to bind bilirubin. In Table II are shown the results of the repeated reuse over a 6-mo period of a single 10-g column to bind bilirubin from 1 ml of jaundiced plasma. This column was tested at well below its capacity in these experiments, but was shown to bind 40 $\mu\text{g}/\text{g}$ of gel when a sample was exposed to an excess of BR at day 192.

We performed a series of experiments with a single batch of agarose-HSA in which a large amount of bilirubin and varying flow rates (0.5–2.5 ml per min) were used in order to stress the gels to near capacity. Part of the gel was stored unused for 3 wk, part was used and eluted only on the day after it was made, and part on the day it was made and then again on days 7 and 14. All portions of the gel were compared on day 20. The binding capacity was only slightly dependent on flow rate over the five-fold range tested. Furthermore, the binding capacity at various flow rates was negligibly changed by either reuse or storage in the ice box.

TABLE I
Necessity of HSA in Binding of BR to Agarose

Gel	BR bound $\mu\text{g}/\text{g gel}$
Sepharose 6B	0.9
Sepharose 4B-HGG	0.3
Sepharose 6B-HSA	71.8

1 ml plasma with 240 μg BR was passed over 3 g agarose-HSA. The amount bound represents the difference between the added BR and the amount recovered with PBS washes.

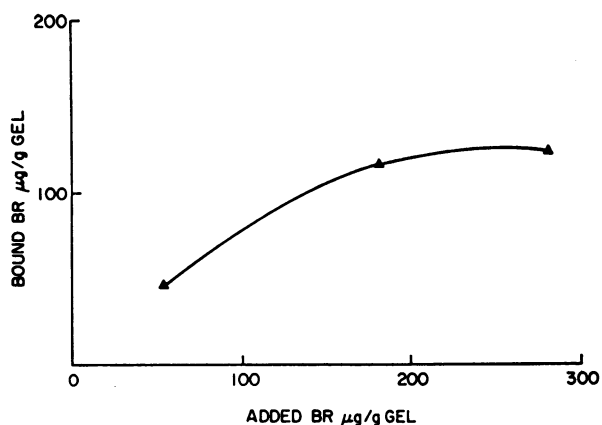


FIGURE 2 Apparent saturation of agarose-HSA. 1, 3, and 5 ml of plasma with 180 μg BR/ml were passed over identical columns of 3 g of agarose-HSA.

In further experiments we have found that elution and storage in 70% (vol/vol) ethanol, chosen because of its potential bacteriostatic properties, was as effective as 50% ethanol and did not decrease the subsequent bilirubin-binding capacity of the beads.

Binding of other substances to agarose-HSA. The results of experiments on the binding and elution of other substances are summarized in Table III.

Thyroxine was partially bound to agarose-HSA. The fraction of the material which bound could not be eluted with PBS but could be eluted with 50% ethanol.

Cortisol was slightly retarded by the beads as evidenced by the fact that a portion appeared in the prolonged PBS wash, but PBS did elute virtually all the material.

Three bile salts were tested. The unconjugated dihydroxy bile salt, chenodeoxycholate, bound strongly and could be eluted with 50% ethanol but not with 1 M sodium chloride-0.1 M sodium acetate, pH 5.0. The conjugated monohydroxy salt, tauroolithocholate, was similarly bound and could be eluted with 50% ethanol. The conjugated trihydroxy salt, taurocholate, was definitely

TABLE II
Effect of Reuse of a 10-g Agarose-HSA Column on the Binding of BR in 1 ml of Plasma

Length of storage days	BR added $\mu\text{g}/\text{g gel}$	BR bound $\mu\text{g}/\text{g gel}$
1	19.3	13.3
7	19.3	14.6
15	19.3	15.3
56	17.8	14.3
91	23.8	20.1
192	23.8	24.1

TABLE III
Interaction of Other Substances with Agarose-HSA

Substance	Amount added	Elution			
		First PBS	Total PBS	1 M sodium chloride 0.1 M sodium acetate, pH 5	50% ethanol
	μg	<i>percent of added compound recovered</i>			
Thyroxine*	Trace	73.2	73.2	ND	27.6
	Trace	77.3	77.3	ND	28.5
Cortisol*	Trace	101	112.2	0.9	1.9
	Trace	95.2	103.7	1.0	1.4
Chenodeoxycholate*	0.915	10.7	10.7	1.0	94.2
	2.19	2.5	10.7	0.2	69.4
Tauroolithocholate*	1.14	33.3	41.2	ND	42.1
	1.05	40.9	48.5	ND	46.7
Taurocholate*	3.46	49.7	93.0	2.3	4.9
	3.81	71.2	105.1	0.8	3.2
Salicylate	183	10.5	96.9	ND	1.3
Digitoxin	0.031	4.0	4.0	1.1	61.5
	0.055	2.7	2.7	1.2	89.2
Digoxin	0.0026	100.2	100.2	0	0
	0.0030	100.0	100.0	0	0

* The additions of thyroxine and cortisol were well below the serum levels of these compounds. Since we did not measure the levels of bile salts, however, the amount added may well be a substantial fraction of the endogenous levels. In each experiment the radioactive compound was equilibrated in 1 ml plasma or serum before passage over 5 g agarose-HSA. ND means not done.

retarded by the beads but did elute with a prolonged PBS wash, presumably indicating a weak ionic or hydrogen bond linkage to the agarose-HSA.

Sodium salicylate resembled taurocholate since it was retarded but eventually eluted completely with neutral buffer.

Digoxin, which does not bind significantly to albumin, and digitoxin, which does (19), were studied at plasma

levels in the ranges associated with clinical toxicity. As Table III shows, no digoxin was retained by the beads. By contrast, virtually all of the digitoxin was retained and was eluted by 50% ethanol but not by 1 M sodium chloride-0.1 M sodium acetate, pH 5.0.

Indocyanine green (not shown in Table III) clearly bound to the beads. It could be eluted by 50% ethanol but not by 1 M sodium chloride-0.1 M sodium acetate, pH 5.0.

Studies with whole blood. The agarose-HSA beads were able to remove bilirubin from whole blood from a patient with the Crigler-Najjar syndrome (Table IV).

We studied the effect on various hematologic and chemical parameters of the passage of fresh normal blood over agarose-HSA columns. When 10 ml of fresh blood anticoagulated with EDTA was passed over 5-g columns, the leukocyte count, differential, and platelet count were virtually unchanged (Table V). The hemoglobin and red blood cell count were unchanged. A number of clotting factors of citrated whole fresh blood were likewise little affected by a single pass over agarose-HSA (Table VI). The erythrocyte osmotic fragility, tested both

TABLE IV
Removal of BR from Whole Human Blood
by Agarose-HSA

Experiment	Plasma BR concentration	
	Initial	Final
	$\mu\text{g/ml}$	
152	244	101
153	283	184

10 ml whole fresh heparinized blood from a patient with the Crigler-Najjar syndrome was passed over 7 g agarose-HSA. BR was measured by the van den Bergh reaction.

before and after incubation at 37°C, was unchanged by the passage of blood over agarose-HSA.

Among a large series of clinical chemistry studies, the following were not significantly changed by passage of whole blood over agarose-HSA: sodium, potassium, chloride, bicarbonate, phosphate, blood urea nitrogen, creatinine, uric acid, glucose, total protein, IgA, IgM, ceruloplasmin, serum glutamic oxaloacetic and pyruvic transaminase, alkaline phosphatase, fatty acids, triglycerides, cholesterol, protein-bound iodine, triiodothyronine, and thyroxine. The calcium fell from 6.6 to 4.7 and from 5.6 to 4.7 mg/100 ml in two experiments, and the magnesium from 1.2 to 0.9 mg/100 ml in one experiment (the column had been equilibrated with PBS containing no divalent cation). Minor reductions in IgG and increases in haptoglobin occurred in two experiments, and the iron and iron-binding capacity fell slightly in one experiment. There was no evident qualitative change in the immunoelectrophoresis of the serum from whole blood passed over an agarose-HSA gel column (Fig. 3).

DISCUSSION

The experiments reported here arose from the need to deal with the declining clinical state of a young woman with congenital nonhemolytic jaundice (the Crigler-Najjar syndrome) who had survived to age 18 before central nervous system signs developed which resembled kernicterus (20). Removing unconjugated bilirubin from her circulation is the only function her otherwise normal liver fails to perform. We considered, therefore, the problem of removing a substance so tightly bound

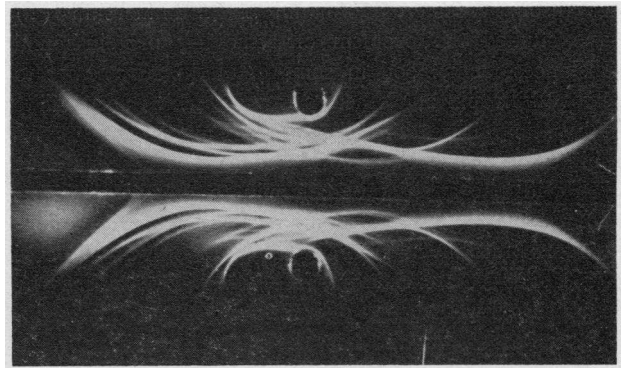


FIGURE 3 Immunoelectrophoresis of serum from whole blood before (above) and after (below) passage over agarose-HSA. 25 ml of heparinized normal blood was passed over 10 g of agarose-HSA and the plasma separated by centrifugation and clotted by the addition of protamine sulfate. The plate was developed with rabbit anti-whole human serum (Dr. H. Metzger).

to albumin that conventional methods of assisting removal are either ineffectual (dialysis) or impossibly cumbersome for chronic use (exchange transfusion and phototherapy).

The reason that protein-bound substances cannot dialyze is not that there is no free compound. Rather, a high binding constant of the compound to the protein implies that a single molecule is statistically unlikely to have a long enough time for free diffusion after dissociating from the protein molecule to cross to and then through a dialysis membrane before meeting and binding to another protein molecule. If there were protein molecules within free diffusion distance of the plasma proteins but immobilized so that they would not move with the moving plasma stream, they ought to compete with the circulating protein molecules for the free compound and so remove the compound from the plasma stream. Eventually, a new steady state would be achieved with equilibrium of compound between free and immobilized protein.

TABLE V
Effect of Agarose-HSA on Leukocyte Platelets

Experiment	Leukocytes		Polymorphonuclear leukocytes		Platelets	
	Pre	Post	Lymphocytes		Pre	Post
			Pre	Post		
	<i>cells/mm³ × 10⁻³</i>		%		<i>cells/mm³ × 10⁻³</i>	
119*	4.5	4.2	$\frac{59}{35}$	$\frac{64}{32}$	232	244
120*	4.35	4.15	$\frac{64}{28}$	$\frac{62}{30}$	223	209
133*	4.5	4.5	$\frac{68}{28}$	$\frac{60}{36}$	234	214
178‡	4.5	1.9	$\frac{43}{50}$	$\frac{41}{57}$		

* 10 ml heparinized blood was passed over 5 g agarose-HSA.

‡ 25 ml citrated blood was passed over 10 g gel. In this experiment, some bubbles appeared in the column because of a loose connection and may account for the fall in leukocytes.

TABLE VI
Effect of Agarose-HSA on Some Clotting Factors

	Experiment 118		Experiment 132	
	Pre	Post	Pre	Post
Fibrinogen (mg/100 ml)	173	165	172	153
Prothrombin (s)	11.4	12.4	13.3	12.8
Partial thromboplastin time (s)	25.8	28.6	30.0	29.6
Thrombin time (s)	28	28	32	33
Factor V (% control)	68	51	53	46
Factor VII (% control)	94	74	54	59

10 ml of fresh citrated blood was passed over 5 g agarose-HSA. The blood was diluted by 20% with citrate-saline during the column passage. A similar dilution as made for the control.

Willson, Webster, Hofmann, and Summerskill (1) have studied the problem of removing protein-bound substances from blood in some detail in an attempt to remove bilirubin and other substances which accumulate in hepatic failure. They found that unconjugated bilirubin could not be dialyzed in a counter-current dialyzer, even with the addition of albumin to the dialysis bath (1). Some drugs or metabolites can increase the dialysance of bilirubin marginally (21), but only at the cost of increasing the circulating free bilirubin which may be responsible for its central nervous system toxicity (22). Willson et al. found that the anion exchange resin Dowex-1 and the neutral resin XAD-2 efficiently removed bilirubin from plasma, and they have pursued the study of resins as a means of treating liver failure and drug intoxication (23). We have confirmed their observations on the binding of unconjugated bilirubin by these resins³ but have decided to develop a system employing specific proteins to bind particular substances because of the perhaps theoretical advantages for blood compatibility and specificity.

In the experiments reported here, we found that it was possible to couple normal HSA, an abundantly available protein, to an insoluble matrix, agarose, in concentrations equal to those in serum; that the bound albumin retained a considerable ability to bind unconjugated bilirubin and other compounds normally bound to albumin; that the bound albumin competed efficiently with serum albumin passed over the gel adsorbent; that some of the substances retained on the gel adsorbent could be removed by elution with physiological salt solution, while others eluted with 50% ethanol in water; that such eluted gels retained their capacity to bind compounds with repeated reuse and storage at ice box temperatures; and that whole blood could be passed over such gels with only minor changes in white blood cells, platelets, clotting factors, and a variety of plasma proteins, electrolytes, and metabolites.

Although we did not measure the binding constant of the agarose-conjugated albumin, it appeared to compete efficiently for the bilirubin bound to circulating albumin and, in *in vivo* experiments, we have obtained some evidence of the achievement of equilibrium during prolonged *in vivo* perfusion (10). The successful elution of the gel-bound bilirubin by plasma or solutions of HSA supports the concept that the binding of the BR to the conjugated HSA is not qualitatively different from the binding to circulating albumin.

Furthermore, the binding of other substances to the conjugated albumin resembles the binding to albumin in solution. Salicylate is relatively weakly bound to albumin (24), and we have found that prolonged washing of the gel with physiological saline can remove the retarded

³ Unpublished observations.

drug. Cortisol, which also binds weakly to albumin (25), behaved similarly. Thyroxine, which in plasma binds largely to a group of specialized binding proteins of high affinity and, to a small extent, to albumin (26), did bind partially to the gel. It would be interesting to know if only the albumin-bound material transferred to the gel.

The binding of bile salts to albumin has been studied in detail by Rudman and Kendall (27). Our findings of weak binding by taurocholate and stronger binding by tauroolithocholate and chenodeoxycholate are in accord with his studies.

The binding of digitoxin to the gels and the failure of digoxin to bind agree well with the studies on digitalis binding to albumin (19) by Smith.

These experiments suggest that it may be possible to develop specific protein adsorbents other than albumin for the removal of particular metabolites or toxins from the circulation. Specific antibodies (28) (perhaps Fab fragments to avoid complement fixation), C1q (to remove circulating immune complexes), and other plasma transport proteins might be usefully attached to polymer beads. Despite our exploratory experiments with a variety of insoluble polymer beads, and despite the apparent biocompatibility of agarose with whole human and rat blood, other insoluble matrices may eventually prove more suitable for human use. Derivatives of methacrylate or other plastics of known blood compatibility may be useful (29).

In the accompanying paper (10), our experience in using agarose-HSA gel adsorbents *in vivo* in rats to remove both conjugated and unconjugated bilirubin is presented and some other practical problems with these gels are discussed.

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