Removing Substances from Blood by Affinity Chromatography

II. REMOVING BILIRUBIN FROM THE BLOOD
OF JAUNDICED RATS BY HEMOPERFUSION
OVER ALBUMIN–CONJUGATED AGAROSE BEADS

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A B S T R A C T In vitro studies indicate that bilirubin and other albumin-bound substances can be efficiently removed from plasma by filtration over albumin-conjugated agarose beads. The effectiveness of this technique in vivo was investigated in rats by using a closed extracorporeal hemoperfusion system. Five Gunn rats whose endogenous bilirubin pool had been labeled with [³H]bilirubin and five Sprague Dawley rats with surgically created biliary obstruction were chosen as models of unconjugated and conjugated hyperbilirubinemia. Indocyanine green was injected into rats and its removal also studied. In the Gunn rats, 98% of the bilirubin was removed from plasma during the initial pass over the column as determined isotopically and chemically. Plasma bilirubin levels fell more than 70% from 8.2 ± 1.6 mg/100 ml (mean$\pm$SD) to 2.6 ± 0.5 mg/100 ml during a 1-h hemoperfusion. An average of 1,061 μg of bilirubin was recovered from the columns, representing $22.5\pm4.2\%$ of the total exchangeable bilirubin pool and $96\pm36.4\%$ of the plasma pool. Results were similar in the rats with biliary obstruction and in those given indocyanine green. Normal Sprague Dawley rats experience minimal changes in formed blood elements, electrolytes, and proteins as the result of hemoperfusion. When the total volume of the column did not exceed 51% of the estimated blood volume of the animal, the survival rate was 100% in 20 studies, and the procedure was without observable ill effect. Extrapolation of both in vitro and in vivo data to man suggests that extracorporeal hemo-perfusion over albumin-agarose columns may be a practical means of assisting hepatic excretory function.

INTRODUCTION

The accompanying paper describes the efficient removal of bilirubin (BR)[1] and other protein-bound substances from plasma and whole blood in vitro by filtration over agarose beads coupled to human serum albumin (HSA) (1). The purpose of the studies reported here was to evaluate the effectiveness of these beads in removing BR from the circulation in vivo when a simple extracorporeal hemoperfusion system was used. Congenitally jaundiced Gunn rats which lack glucuronyl transferase and rats with surgically created biliary obstruction were chosen as models of unconjugated and conjugated hyperbilirubinemia, respectively. Indocyanine green (ICG), another tightly albumin-bound substance, was injected into rats and its removal also studied. In addition, the effect of hemoperfusion on formed blood elements and plasma electrolytes was examined.

METHODS

Columns. In most of the experiments, Pharmacia K9/30 columns (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were used. Some of these columns were modified by cutting and rethreading the plastic barrel to decrease the column volume and still allow use of the original ends. The bed supports and nylon mesh supplied with the column

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[1] *Abbreviations used in this paper:* BR, bilirubin; {BR}, bilirubin concentration; C_{BR}, bilirubin clearance; HSA, human serum albumin; ICG, indocyanine green.

were replaced at both the top and bottom ends by plain or gold-plated (Alexandria Metal Finishers Inc., Alexandria, Va.) stainless steel cloth (200 mesh). Disposable polypropylene columns (Chromaflex, Kontes Glass Co., Vineland, N. J.) with nylon cloth ends (400 mesh) and Pharmacia K16 glass barreled columns treated with silicone (Dow Corning #360 medical fluid, Dow Corning Corp., Midland, Mich.) were also used.

Agarose-albumin conjugates. Sepharose 6B (Pharmacia Fine Chemicals, Inc.) or Biogel A5m, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.) was coupled to HSA by methods described in the previous paper (1). The agarose-HSA conjugates contained 30–50 mg HSA per gram wet wt of gel.

Packing was accomplished by pouring a slurry of agarose-HSA into a column containing a small amount of 0.14 M sodium chloride and regulating the outflow at 1 cm^3/min with an LKB Series 12,000 peristaltic pump (LKB Industries, Bromma, Sweden). When the column was fully packed and the upper retaining mesh put in place, air was expelled from the upper end by gently tapping the column while 0.14 M sodium chloride was pumped into the lower end.

Extracorporeal hemoperfusion technique. The rat to be hemoperfused was anesthetized with a 50 mg per kg intraperitoneal injection of pentobarbital (Abbott Laboratories, Chicago, Ill.) and an artery and vein cannulated with PE 50 tubing (Clay-Adams, Inc., Parsippany, N. J.). Although the right carotid artery and jugular vein were used in most instances, the femoral vessels were also used in animals undergoing multiple studies. The arterial cannula was connected to a modified Teflon stopcock which served as an inflow sampling site (2). This, in turn, was connected to the top end of the column containing the gel. Interposed between the bottom end of the column and the venous cannula were an LKB peristaltic pump which regulated the flow of blood in the circuit and a second (outflow) sampling site. The complete extracorporeal hemoperfusion circuit is shown in Fig. 1.

The extracorporeal circuit was primed with warmed heparinized saline (2 U/cm^3), and the rat given 500 U/kg body wt of heparin intravenously. The arterial and venous cannulae were then connected and the pump started. Blood flow through the extracorporeal circuit was generally maintained at 1 cm^3 per min. During the study, the rat's body temperature was monitored by a rectal thermometer and maintained at 36–37°C with an infrared lamp. Control blood samples for determination of plasma bilirubin concentration ({BR}) were obtained from the inflow sampling site at the beginning of the study. After a quantity of blood equivalent to the total volume of the column had passed through the circuit, a second sample was taken from the outflow sampling site. When compared with the control values, this sample allowed calculation of the percentage of BR removed by the gel during the initial pass. Perfusion was generally continued for 1 h, and a blood sample was taken from the outflow sampling site just before termination of the study. At the completion of hemoperfusion, the blood remaining in the circuit was returned to the rat by flushing a single column volume of warmed, heparinized saline through the system. A final sample of rat blood was then taken from the arterial line for determination of plasma {BR}. Comparison of these last two samples indicated whether the gel had equilibrated with the plasma or was still removing BR at the end of the study. The samples obtained from the rat before and after hemoperfusion indicated the overall
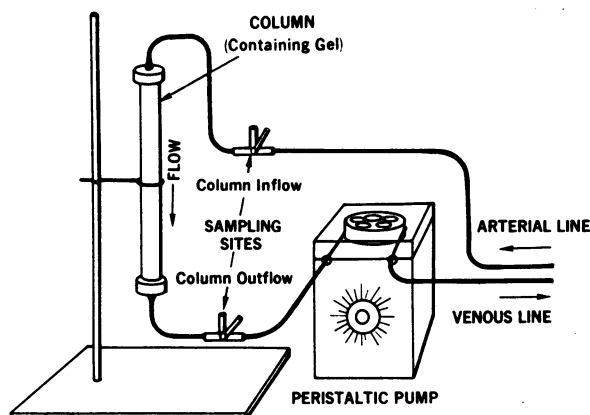

FIGURE 1 Extracorporeal hemoperfusion circuit.

effectiveness of the procedure in lowering the plasma {BR} in vivo.

At the completion of perfusion, the arterial and venous cannulae were removed and the rat allowed to awaken. The column was washed with two column volumes of phosphate-buffered saline (0.14 M sodium chloride–0.01 M sodium phosphate, pH 7.2) and then with one column volume of 1.0 M sodium chloride in the reverse direction. Elution with 50% (vol/vol) ethanol in water was then performed as described (1), and the ethanol eluate assayed for BR and radioactivity.

Bilirubin measurements. The {BR} in rat plasma was determined on 20-μl samples by using a micromodification of the method of Weber and Schalm (3). In the Gunn rat studies, the total {BR} in the ethanol eluate obtained from the columns was determined by three different methods. (*a*) The van den Bergh reaction was used as described in the in vitro studies (1). (*b*) An aliquot of the ethanol eluate was diluted 1:2 in 4% HSA and the optical density at 454 nm measured. The {BR} was then determined by comparing this value with standard curves obtained from known concentrations of BR dissolved directly in a 2% HSA–25% ethanol solution. (*c*) A 1:5 dilution of the eluate in 5% HSA was subjected to the partition method of Weber and Schalm (3). With this dilution of the eluate, only one part in 96 of the total partition mixture was ethanol, and virtually all (96±5.6%, mean±SD) of the unconjugated BR partitioned into the lower, nonpolar layer. Values obtained by these three methods agreed within an average of ±4.6% of the mean and were averaged in the final results.

The total {BR} in the ethanol eluates obtained from the rats with biliary obstruction was measured by the van den Bergh reaction, and the percentage of this total representing conjugated BR was determined by the Weber-Schalm partition.

Indocyanine green measurements. The ICG concentration was determined in rat plasma by comparing the optical density at 805 nm with standard curves in rat plasma. The 50% ethanol eluates were diluted 1:2 in 4% HSA and the optical density compared with standard curves of ICG dissolved in 2% HSA–25% ethanol.

Animal preparation. A total of five studies was performed on four homozygous, jaundiced Gunn rats obtained from the Animal Resources Branch of the National In-
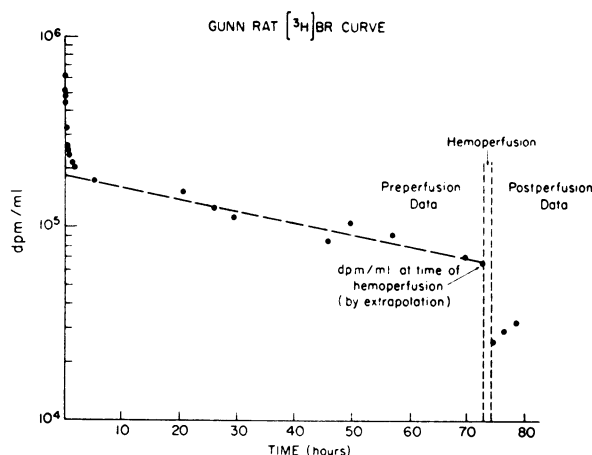
GUNN RAT [³H]BR CURVE

FIGURE 2 [³H]BR disappearance curve in a jaundiced Gunn rat. The ordinate represents plasma radioactivity present in the lower, nonpolar layer of the Weber Schalm partition. The vertical dashed lines separate data obtained before and after the 1-h hemoperfusion.

stitutes of Health. Anesthetized Gunn rats were given an average of 6.6 ± 0.6 μCi (54 ± 5 μg) of [³H]BR, prepared in bile fistula dogs as previously described (4). By using a special sampling apparatus (2), arterial samples were obtained every 30 s for the first 3 min and at longer intervals thereafter. At 75 min, the cannulae were removed and the animals allowed to awaken. Subsequent blood samples before extracorporeal hemoperfusion were obtained from a lateral tail vein over the next 72 h after which the animals were hemoperfused as described above. All plasma samples were subjected to a micromodification of the Weber-Schalm partition and the radioactivity extracted into the lower layer was used for calculation of the plasma isotope disappearance curve. The procedure is analogous to that previously described (5). A typical [³H]BR disappearance curve in a jaundiced Gunn rat is shown in Fig. 2. Plasma BR pool and total miscible BR pool were calculated from the radiobilirubin disappearance curves and the plasma {BR} as indicated below.

Radioactivity present in the injected material, the ethanol eluate from the perfused column, and the lower layer of the Weber-Schalm extraction of plasma samples were determined by standard liquid scintillation counting methods in Aquasol (New England Nuclear, Boston, Mass.), with internal standardization for quench correction. Multiple aliquots of the ethanol eluate were counted, and at least 5,000 counts were accumulated on all samples and background in order to insure that the error in the net count rate did not exceed 3%.

To prepare the biliary obstruction rats, five normal Sprague Dawley rats were anesthetized and their bile ducts ligated through a small laparotomy incision. The plasma {BR} generally plateaued in 3–5 days and the animals were hemoperfused 1–10 days thereafter.

In preparation for the ICG studies, two Sprague Dawley rats were anesthetized and their bile ducts ligated. They were given 35 mg/kg ICG (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) intravenously and frequent arterial samples were obtained until a relatively stable plasma ICG concentration was reached. The animal was then hemoperfused.

Laboratory determinations. Blood electrolytes and calcium concentration and complete blood counts including platelet counts and leukocyte differential counts were performed on coded samples (Bionetics Medical Laboratories, Falls Church, Va.). Peripheral blood smears before, during, and after hemoperfusion were examined by the investigators. Plasma cortisol determinations were performed by radioimmunoassay in the laboratory of Dr. Lynn Loriaux at the National Institutes of Health. Total plasma thyroxine levels were determined by competitive protein binding in the National Institutes of Health clinical laboratories.

Other studies. An additional 23 normal Sprague Dawley rats were hemoperfused with columns of various volumes. Blood samples from the animals before and after perfusion in addition to samples of column effluent were examined for formed blood elements and blood chemistries.

Calculations. In the Gunn rat, the time required for distribution of the injected isotope between the various exchanging body pools ($\sim$90 min) is relatively short when compared with the time required for ultimate disappearance of the isotope from the system (average biological half life = 39 h). This permits analysis of the radiobilirubin disappearance data in terms of a unicompartmental model. Under this circumstance extrapolation of the final exponential to zero time (T_0) yields a reasonable estimate of the total volume of distribution of the injected isotope (TVDBR),[3] and hence the total size of the readily exchangeable bilirubin pool (TBRP = TVDBR $\times$ plasma {BR}. Extrapolation to T_0 of the portion of the curve defined by the initial 3–4 points, obtained during the first 1–2 min after injection of the isotope, yields an approximation of the plasma volume of distribution of the injected [³H]BR (PVDBR) and hence, of the plasma bilirubin pool (PBRP = PVDBR $\times$ plasma {BR}). The plasma [³H]BR activity at the start of hemoperfusion (dpm/ml$_H$) was determined by extrapolating a least squares fit of the final exponential to the time of initiation of hemoperfusion. The plasma {BR} in micrograms per milliliter ({BR}$_P$) used in the calculation of the preperfusion pool sizes is the average of the values obtained 3 h before and at the onset of hemoperfusion. These two figures agreed quite closely. With these data, as well as the total radioactivity (dpm$_{col}$) and micrograms of bilirubin (BR$_{col}$) recovered from the column, the following calculations are possible.

1. Percentage of TBRP and PBRP removed during hemoperfusion as determined by column isotope recovery

$$\%PBRP = \frac{dpm_{col}}{(dpm/ml_H) \times (PVDBR)_{ml}} \times 100 \quad (1\,a)$$

$$\%TBRP = \frac{dpm_{col}}{(dpm/ml_H) \times (TVDBR)_{ml}} \times 100 \quad (1\,b)$$

2. Percentage of TBRP and PBRP removed as determined by the number of micrograms of bilirubin recovered

[3] *Abbreviations used in these calculations and Table I:* BR$_{col}$, bilirubin recovered from the column; {BR}$_{1P}$, {BR} in column effluent after first pass; {BR}$_P$, plasma bilirubin concentration at the beginning of hemoperfusion; dpm$_{col}$, radioactivity recovered from the column; dpm/ml$_H$, plasma [³H]BR activity in the rat at the beginning of hemoperfusion; dpm/ml$_{1P}$, radioactivity in column effluent present after first pass through circuit; PBRP, plasma bilirubin pool; PVDBR, plasma volume of distribution of [³H]BR; TBRP, total miscible bilirubin pool; TVDBR, total volume of distribution of [³H]BR.

TABLE I
TABLE I
Removal of Bilirubin from Gunn Rats by Extracorporeal Hemoperfusion

Parameter*	Individual Studies					Mean	SD
	1	2	3	4 a	4 b		
Weight (g)	417	400	427	360	415	404	
PVDBR/100 g (ml)	4.9	4.7	3.4	3.8	4.7	4.30	0.59
TVDBR/100 g (ml)	16.3	17.3	17.8	19.1	15.2	17.14	1.32
Biological T½ of [³H]BR	51.3	52.5	34.0	31.5	26.4	39.14	10.71
{BR} (mg/100 ml)							
Plasma, preperfusion	8.2	9.5	10.3	7.6	5.6	8.24	1.62
Plasma, postperfusion	2.6	2.5	3.5	2.1	2.4	2.62	0.47
Column outflow after 1st pass	0	0.5	0	0		0.12	0.22
Column outflow at end of study			3.5		1.9		
% BR removed$_{ip}'$	100	94.7	100	100		98.7	2.6
% BR removed$_{ip}$	99.8	88.9	100	100	98.9	97.5	4.3
BR eluted from column							
Total (μg)	850	1,279	1,325	1,185	667	1,061	258
Per g gel (μg/g)	68.0	102.3	106.0	94.8	53.4	84.9	20.6
BR specific activity							
Rat plasma pre perfusion	662	733	322	450	574	548	147
Column eluate	705	893	535	495	586	643	143
% Total body pool removed							
TBRP (%)	16.2	23.6	28.1	25.2	19.3	22.5	4.2
TBRP' (%)	15.2	19.4	16.9	22.9	18.9	18.7	2.6
% Plasma pool removed							
PBRP (%)	55.0	86.8	148.6	127.1	62.5	96.0	36.4
PBRP' (%)	51.6	71.2	89.4	114.4	61.3	77.6	22.3

* See Methods, footnote 2, for explanation of symbols.

from the column

$$\%PBRP' = \frac{(BR_{col})\mu g}{(PBRP)\mu g} \times 100 \qquad (2\,a)$$

$$\%TBRP' = \frac{(BR_{col})\mu g}{(TBRP)\mu g} \times 100 \qquad (2\,b)$$

3. Specific activity of [³H]BR recovered from the column

$$\frac{dpm_{col}}{(BR_{col})\mu g} \qquad (3)$$

4. Specific activity of [³H]BR in the rat at the time of hemoperfusion

$$\frac{dpm/ml_H}{(\{BR\}_p)\mu g/ml} \qquad (4)$$

In addition, the isotope data and chemical measurements of BR obtained from the column outflow site after a single column volume of blood had passed through the perfusion circuit (dpm/ml_{ip}, $\{BR\}_{ip}$) permit calculation of the percentage of BR removed during the initial pass over the column (%BR removed$_{ip}$).

5. Percentage removed as determined by isotope data

$$\%BR\ removed_{ip} = \frac{(dpm/ml_H) - (dpm/ml_{ip})}{(dpm/ml_H)} \times 100 \qquad (5)$$

6. Percentage removed as determined by chemical measurements

$$\%BR\ removed_{ip}' = \frac{(\{BR\}_p) - (\{BR\}_{ip})\mu g/ml}{(\{BR\}_p)\mu g/ml} \times 100 \qquad (6)$$

RESULTS

Studies of Gunn rats. Five extracorporeal hemoperfusions were performed on four Gunn rats, and results of these studies are summarized in Table I. Columns 4 a and 4 b represent replicate studies on the same animal.

In only one experiment was any BR detectable in the column effluent by direct chemical measurement after a single pass through the column, and in this study the {BR} fell from 9.5 mg/100 ml to 0.5 mg/100 ml. [³H]BR data also indicate that virtually all (97.5±4.3%) of the bilirubin was removed from this first column volume of blood during the initial pass through the circuit. During the hour of hemoperfusion, plasma {BR} in the rats fell more than 70% from 8.24±1.62 mg/100 ml to 2.62± 0.47 mg/100 ml. By chemical measurement, an average of 1,061 μg of BR was recovered from the ethanol column
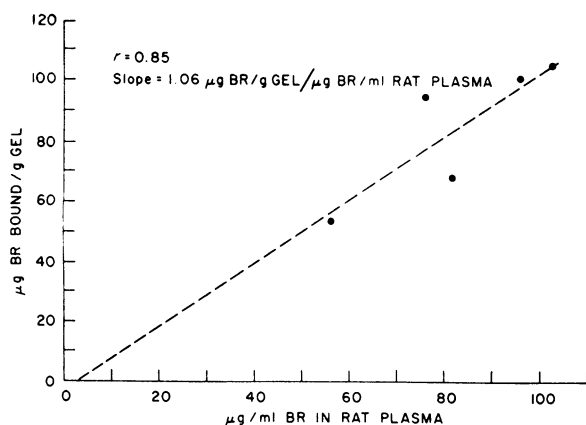
FIGURE 3 Relationship between the amount of unconjugated BR bound to the column expressed as μg/g gel hemoperfusion (ordinate) and the {BR} in the rat before hemoperfusion (abscissa).

eluates. This represents 77.6±22.3% of the initial preperfusion plasma bilirubin pool and 18.7±2.6% of the total body pool (%PBRP' and %TBRP'). Analogous calculations based on measurements of radioactivity recovered from the column (%PBRP, %TBRP) show that 96.0±36.4% of the plasma bilirubin pool and 22.5±4.2% of the total miscible pool were removed. The fact that

{BR} did not drop to zero in those studies in which a quantity of BR equal to the entire PBRP was removed probably indicates mobilization of extravascular pools.

The specific activity of the BR recovered from the 50% ethanol eluates agreed quite closely in four of the five studies with the calculated specific activity of the BR in the rat before hemoperfusion. There was generally a several hour delay between the end of the perfusion and the chemical measurement of the BR in the ethanol eluate. Bilirubin is a highly light-sensitive compound and some photodegradation during this period may explain the slightly higher specific activity of BR recovered from the column when compared with that in the rat. In study 3 (Table I), technical problems with column elution resulted in a significant underestimate of the BR recovered from the column, as measured chemically. This may explain the greater discrepancy in specific activities observed in this particular study, as well as the fact that %TBRP' and %PBRP' determined chemically did not agree with corresponding values determined isotopically (Table I).

A 12.5-cm³ column was used in all Gunn rat studies. The column appeared to have equilibrated with plasma at the end of each 60-min perfusion. This conclusion was based on two observations. First, the column was uniformly yellow-orange from top to bottom. Second, the

TABLE II

Removal of Bilirubin from Biliary Obstruction Rats by Extracorporeal Hemoperfusion

Parameter	Individual studies							Mean	SD
	1	2 a	2 b	3 a	3 b	4	5		
Weight (g)	560	655		665		750	545	635	
Duration of study (min)	60	45	45	60	60	60	60	55.7	
Quantity of gel (g)	12.5	18	20	18	21.5	21	18	18.4	
{BR} rat, pre									
Total (mg/100 ml)	6.3	8.9	7.4	8.1	6.3	6.7	8.6	7.47	1.0
Conjugated (% of total)	86	92	93	91	92	100	88	91.7	4.1
{BR} rat, post									
Total (mg/100 ml)	5.0	5.1	4.7	6.3	3.9	4.1	5.1	4.88	0.73
Conjugated (% of total)	88	100	100	92	95	98	86	94.1	5.2
{BR} column outflow (mg/100 ml)									
After initial pass	0.1	0.3	0.6	0	0.7	0	0	0.24	0.28
At end of study	1.7	1.0	1.1	1.6	0.9	1.4	1.2	1.27	0.28
BR removed by gel (%)									
BR removed$_{ip}$	98.4	96.6	91.9	100	88.9	100	100	96.54	4.1
At end of study	66.0	80.4	76.6	74.6	76.9	65.8	76.4	73.81	5.25
BR eluted from column									
Total (μg)	1,160	930	898	1,250	847	1,080	974	1,020	137
Conjugated (% of total)	88	94	90	91	87	100	87	91.0	4.3

{BR} in the column effluent just before the termination of hemoperfusion and the {BR} in the rat after hemoperfusion agreed quite closely (Table I, studies 3 and 4 *b*), suggesting that the column was no longer removing BR from the circulating rat blood at the end of the study. Together, these suggest that the amount of BR bound to the gel should reflect, at least in part, the initial plasma {BR} in the rat. When the quantity of bilirubin removed (μg/g gel) is plotted as a function of the initial plasma {BR} (μg/ml) (Fig. 3), a linear relationship is observed. Although the five Gunn rat studies represent a relatively narrow range of plasma BR concentrations, (5.6–10.3 mg/100 ml), the slope of the regression line (1.06) and the correlation coefficient ($r = 0.85$) suggest that the column functions in vivo very much as it has in vitro (1). Furthermore, the data suggest that in these experiments the column capacity (up to 106 μg/g gel) was limited primarily by the initial {BR} in the rat.

Biliary obstruction rats. Seven hemoperfusions performed on five rats with biliary obstruction are summarized in Table II. The data from two rats undergoing replicate studies are shown in columns 2 *a* and *b* and 3 *a* and *b*. Unlike the Gunn rat, whose replicate studies were 3 wk apart, these were completed within several hours of each other.

Virtually all (96.5%) of the predominantly conjugated BR in the obstructed rat studies was removed from the first column volume of blood passing through the gel. In addition, the percentage of total BR recovered from the column after hemoperfusion which was conjugated (91.0%) was nearly identical to the percent conjugated BR present in the rat before hemoperfusion (91.7%). In the rats with biliary obstruction and the Gunn rats the flow rate through the extracorporeal circuit (1 cm³/ min in all studies), the average BR concentration before hemoperfusion (7.47 and 8.24 mg/100 ml) and therefore the total BR load presented to the column, the total time of hemoperfusion (55.7 and 60 min), and the total amount of BR removed from the column (1,020 and 1,061 μg) were very similar. It thus appears that the albumin-agarose beads bound conjugated BR just as effectively as unconjugated.

The overall fall in {BR} in the rats with biliary obstruction after hemoperfusion (from 7.47 to 4.88 mg/100 ml) was less than that in the Gunn rats. However, because of the significantly larger size of the rats with biliary obstruction (635 vs. 404 g), the total number of plasma volumes passed through the column was less and the plasma and total miscible BR pools were greater in this group than in the Gunn rats.

Inspection of the columns following hemoperfusion showed them to be much more yellow-orange at the top than at the bottom, suggesting that complete equilibration with plasma had not yet occurred. This observation was confirmed by comparison of the average {BR}

in the rat after hemoperfusion (4.88 mg/100 ml) and in the column effluent at the termination of hemoperfusion (1.27 mg/100 ml) which indicates that, at the end of the study, the column was still removing nearly 75% of the BR presented to it. This contrasts with the nearly complete equilibration of BR between the rat plasma and gel which occurred in the Gunn rat studies and probably reflects the fact that bigger columns were used in the rats with biliary obstruction (mean 18.4 vs. 12.5 ml).

Column elution. In both the Gunn and biliary obstruction rat experiments, essentially all the BR as determined chemically and all the radioactivity recovered from the column was contained in the 50% ethanol eluate. No BR or radioactivity was eluted with either the phosphate buffered saline or 1.0 M sodium chloride washes.

The 50% ethanol solution eluted both conjugated and unconjugated BR in a sharp band and returned the gel to a translucent state. The gel following ethanol elution in the Gunn rat studies was indistinguishable from freshly prepared gel. After elution of the predominantly conjugated BR in the biliary obstruction studies, however, the gel appeared a faint green. Elution with up to 70% ethanol did not remove this green color.

Indocyanine green rats. ICG, like conjugated and unconjugated BR, was nearly completely cleared (average = 97.3%) from the first column volume of blood on its initial pass through the circuit, and an average of 1,016 μg of ICG was recovered from the ethanol eluate. The plasma ICG concentration decreased markedly after hemoperfusion in both studies (from an average of 23.7 mg/100 ml before hemoperfusion to 10.7 mg/100 ml after hemoperfusion), although approximately 10–15% of this decrease may be attributed to continued hepatic clearance, since the ICG concentration had not quite plateaued when hemoperfusion was begun.

As in the studies with BR, no ICG was eluted with either phosphate buffered saline or 1.0 M sodium chloride. The 50% ethanol solution eluted the ICG in a sharp green band and restored the gel to its pre-perfusion translucent state.

Additional data. Samples taken from rats before and after hemoperfusion, as well as from the column effluent, were examined for formed blood elements. Table III includes data from two representative studies. The hematocrits generally remained unchanged or decreased slightly, probably as a result of surgical blood loss, heparinization, blood sampling, and hemodilution. Erythrocyte morphology was unchanged. Platelet counts in the intact animal generally showed a modest decrease during hemoperfusion, and samples taken directly from the column effluent varied from unchanged to markedly decreased. Total leukocyte counts in the intact animal

TABLE III
TABLE III
Hematological Effects of Extracroporeal Hemoperfusion

| | Hematocrit | Hemlglobin | Platelets | White cells | Differentials | | |
					Polys	Lymphs	Monos
	%	g/100 cm³	× 10³/mm³	per mm³		%	
Study 21							
Rat, pre	48.5	16.1	312	6,400	36	64	
Column outflow (1st pass)	41.5	14.4	15	2,100	9	89	2
Rat, post	44	14.7	217	11,000	51	45	2
Rat, 2 h post	42	14.2	317	17,300	82	18	
Study 24							
Rat, pre	38.5	14.2	538	13,800	34	59	7
Column outflow (1st pass)	32.5	12.1	307	5,300	2	97	1
Rat, post	38.5	14.2	459	12,200	51	45	2

either remained unchanged or increased, with a relative increase in the number of young forms. Samples obtained directly from the column effluent generally showed a selective loss of granulocytes, and granulocytes were found in the spun sediment from saline washes. Inspection of the gel and column components suggested that much of the loss of granulocytes and platelets which did occur was caused by trapping in the nylon or stainless steel cloth used to retain the gel, and not by the gel itself.

Two studies revealed no significant changes in plasma electrolytes or protein electrophoresis, and calcium decreased slightly from 10.8 to 10.1 mg/100 ml and from 9.8 to 9.2 mg/100 ml on two occasions. In three studies, plasma cortisol concentration in the rat decreased from 17.85 ± 0.57 μg/100 ml before perfusion to 12.70 ± 0.82 μg/100 ml after perfusion. The cortisol concentration in the column effluent was 0.21 ± 0.29 μg/100 ml after a single column volume of blood had passed through the system and 17.21 μg/100 ml at the end of hemoperfusion (single study). Similar results were found in a single study of plasma thyroxine, which decreased from 8.1 to 1.7 μg/100 ml in the rat following hemoperfusion with no detectable thyroxine in the column effluent after a single pass. Thyroxine levels in the column effluent at the end of the study were not measured. Although these results might suggest that cortisol, like BR, is tightly bound to the beads, they are also compatible with simple retardation of cortisol by the albumin-agarose beads, as found in vitro (1). The fact that the cortisol concentration in the column outflow at the end of the study exceeded the simultaneous concentration in the rats supports the latter interpretation.

Survival data. A total of 34 animals have undergone 37 hemoperfusions. Four studies have been excluded from survival calculations. One involved a rat with biliary obstruction who died 24 h after hemoperfusion

and 14 days after creation of the biliary obstruction. 2 wk has been the approximate lifespan of several animals with biliary obstruction who were not hemoperfused. The two rats given ICG also died shortly after hemoperfusion, but two similar animals with biliary obstruction given the same dose of ICG but not hemoperfused also died. In the fourth study, the rat died of respiratory obstruction due to a mucous plug which was verified at autopsy.

In the remaining 33 hemoperfusions, 24 animals awoke shortly after completion of the study and remained well for at least a week before being sacrificed. This is an overall survival rate of 73%. The total length of hemoperfusion (up to 2 h), estimated number of blood volumes passed over the column (up to 4), and the animal's weight (200–900 g) did not influence survival. However, it was noted retrospectively that in the 13 studies (five in Gunn rats, eight in Sprague-Dawley rats) in which the column volume exceeded 51% of the estimated blood volume of the animal (estimated blood volume = 6% × body weight), only four animals lived (one Gunn rat, three Sprague-Dawley rats). No attempt was made to prime the extracorporeal circuit with blood, plasma, or other volume expanders. It is probable, therefore, that the animals in this group, when drained of more than half their blood volume and given only an equal volume of saline in return, died of circulatory failure. In the remaining 20 studies, in which the column volume was less than 50% of the estimated blood volume, all the animals lived and the procedure was without observable ill effects.

DISCUSSION

A safe and effective method for the removal of protein-bound drugs and toxic metabolites from the body would find application in a number of clinical situations includ-

ing drug intoxication and hepatic failure. A relatively new approach to this problem involves the adsorption of these substances onto insoluble particles or surfaces.

In the earliest experiments, ion exchange resins were administered by mouth or employed in extracorporeal hemoperfusion systems (6, 7). Various resin types have successfully removed protein-bound substances such as bromosulfophthalein, chenodeoxycholate, and unconjugated BR in vitro (8). Further evaluation of this technique in vivo (9) has shown that bromosulfophthalein can be cleared effectively from plasma, while chenodeoxycholate and conjugated BR are less efficiently removed. ICG was not removed to any significant degree by the resins tested. Resin polymers can also effectively adsorb ammonia (10–14) and a limited trial of resin hemoperfusion in three patients with hepatic coma did significantly lower blood ammonia although clinical improvement was minimal and short-lived (15). Resin hemoperfusion has also been applied to the treatment of drug intoxication, and its use in the treatment of glutethimide and phenobarbital poisoning has been encouraging (16, 17).

These synthetic adsorbents vary widely in their ability to bind test compounds, and have the disadvantage of nonspecific binding of a variety of other plasma components. Thus, significant hypokalemia (12, 18) and hypocalcemia (19) have resulted from perfusion over charged ion exchange resins, although this problem may be circumvented by using a mixture of resins equilibrated with different counter ions (12, 13). Thrombocytopenia and leukopenia (9, 16) have also been complications during in vivo studies.

Activated charcoal readily adsorbs water-soluble wastes such as creatinine, uric acid, and guanidine derivatives (20, 21). The problems of blood compatibility and particle emboli have been largely overcome by microencapsulation with nylon, methacrylate, or cellulose nitrate (22–24), and hemoperfusion over microencapsulated charcoal has been effective in the clinical management of the uremic syndrome (25). Drugs such as acetylsalicyclic acid, acetaminophen, barbiturates, and glutethimide, which show some protein binding, have also been removed from plasma in vitro (26, 27) and in vivo (28–32) by this technique.

Charcoal has been minimally effective, however, in removing BR, which is tightly albumin bound (20, 33). In a single case, attempts to assist hepatic excretory function in a patient with hepatic coma by hemoperfusion over albumin-coated microencapsulated charcoal was encouraging, although no change in blood ammonia and only small changes in {BR} were reported (34). It is possible that the albumin coating of these microcapsules removed BR independently of the charcoal core.

The use of HSA-agarose conjugates has two potential advantages compared to synthetic resins and activated charcoal. The first is the predictable and selective removal only of those substances for which albumin serves as a carrier molecule. Second, the HSA-agarose beads appear to produce no significant changes in formed blood elements. The experiments described here demonstrate the efficient removal of the tightly albumin-bound substances, conjugated and unconjugated BR, and ICG from anesthetized rats by hemoperfusion over these beads.

There is evidence to suggest that rat albumin may bind unconjugated BR less tightly than does HSA (35). However, since the albumin-agarose beads were as effective in removing unconjugated BR from human plasma in vitro (1) as rat plasma in vivo, it is unlikely that any species differences in albumin-binding affinities explains the effectiveness of these beads in this study.

It is possible to calculate the BR clearance (C_{BR}) achieved by the gel in these studies as follows:

$$C_{BR} = (\text{flow rate}) \times (\% \text{ BR removed}) \qquad (7)$$

where the percentage of BR removed by the column is calculated as described above (Eqs. 5 and 6). Since the BR was essentially completely cleared from blood during the first pass over the column in all studies, C_{BR} was approximately 1 cm^3/min initially, and was limited only by the flow rate in the extracorporeal circuit. In the rats with biliary obstruction, where columns of greater bed volume were used, C_{BR} was still ~0.75 cm^3/min at the termination of the study. These values compare favorably with preliminary results indicating that hepatic BR clearance in normal Sprague Dawley rats is 0.30±0.02 cm^3/min/100 g body weight (36).

Laboratory data obtained from the rats before and after hemoperfusion indicate that the intact rat experiences minimal or no changes in the basic blood chemistries measured, or in formed blood elements during hemoperfusion. When the column volume did not exceed 51% of the estimated blood volume, the survival rate was 100%.

Although potential problems such as sterility, pyrogenicity, particle emboli, and hemodynamics must be resolved, it is nevertheless interesting to speculate on the clinical feasibility of this approach for assisting hepatic excretory function. A 70-kg man with a normal red blood cell volume and lifespan produces ~1,900 mg of BR/wk. If it is assumed that (a) he is totally unable to clear this endogenous BR load, (b) his average plasma {BR} before extracorporeal hemoperfusion is 10 mg/100 ml, and (c) an extracorporeal flow of 100 cm^3/min can be maintained (easily achieved with arterio-venous shunts currently in use), it would be possible to achieve an initial C_{BR} of ~100 cm^3/min (10 mg/min) over a fresh column. In comparison hepatic C_{BR} in normal man is 47±10 cm^3/min (5). At a clearance rate of 100 ml/min, an entire week's production of

BR could be removed in slightly over 3 h. In practice, a longer period would certainly be required since C_{BR} will decrease as the column approaches equilibration, and the rate of BR removal (milligrams per minute) will fall as the plasma concentration declines. Furthermore, columns would need to be changed so that one could be eluted and regenerated while another was in use. Even if this results in a quadrupling of the time required to completely remove a week's endogenous BR production, the total of 12 h/wk compares favorably with that of standard hemodialysis programs for patients with renal failure. In more acute situations such as drug overdosage, hepatic coma, and neonatal jaundice, this approach may be more effective and perhaps less cumbersome and hazardous than exchange transfusion.

Extracorporeal hemoperfusion over agarose bound albumin gels may thus be a practical means of assisting hepatic excretory functions in selected situations. Furthermore, use of specific binding proteins other than albumin may allow application of the general principle to a variety of clinical problems.

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