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Agarose-Encapsulated Adsorbent Beads for Direct Hemoperfusion: Preparation and *in Vitro* Evaluation[1)]

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Agarose beads containing a powdered charcoal or a cation-exchange resin were prepared by dropping a hot aqueous suspension of adsorbents in agarose sol into an organic solvent mixture consisting of cyclohexane and chloroform (2:1). The beads were crosslinked for intended use in direct hemoperfusion circuits as adsorbent columns. Their *in vitro* adsorption characteristics for selected adsorbates such as theophylline, paraquat, cholate, *etc.*, from rabbit blood as well as from Ringer's solution were evaluated by means of batchwise adsorption experiments and by the column method. Adsorption rate rather than capacity was reduced by incorporation of the powdered adsorbent into agarose gel, particularly for larger adsorbates. On the basis of equal adsorbent weight, the cation-exchange resin beads adsorbed paraquat from Ringer's solution better than did coated petroleum-based activated carbon beads obtained from a commercial hemoperfusion column. No significant changes in the levels of plasma components were observed after contact with the blood. The degree of platelet reduction was similar for the charcoal beads and control agarose beads without an adsorbent, though that for the resin beads was slightly greater. These adsorbent-containing agarose beads are likely to be of value for blood purification.

Keywords——agarose bead; agarose-encapsulated powdered adsorbent; hemoperfusion; charcoal; activated carbon; cation-exchange resin; paraquat adsorption; blood purification

Introduction

The technique of direct hemoperfusion (DHP) for blood purification was introduced by Muirhead and Reid[2)] in 1948 employing a resin as the adsorbent. Later Yatzidis[3)] reported the use of charcoal columns. Although DHP is more detrimental to blood corpuscles than any other procedure, it is the simplest technique of all blood purification procedures and DHP columns find use either for treating drug overdose or in extracorporeal circuits such as for hemodialysis, hemofiltration, or plasmapheresis.

The adsorbent columns commercially available at present are predominantly of charcoal type, although columns of nonionic resins are also marketed for hemoadsorption.[4)] The initial difficulties associated with charcoal columns such as the leucocyte and platelet loss and embolism due to charcoal dust have been substantially overcome by the introduction of thermoplastic petroleum pitch as a raw material, which can be made into hard spherical beads without binders. The hemocompatibility of the beads is improved by coating them with a blood compatible membrane such as poly(2-hydroxyethyl methacrylate), cellulose, *etc.*[5)] This type of charcoal column is presently considered most satisfactory for adsorption of small molecules, with adequate capacity and rate of adsorption. However, for larger adsorbates and/or highly albumin-bound species such as bile salts, bilirubin, *etc.*, the rate and thus the efficiency of adsorption are substantially reduced.

The use of enlarged agarose beads for DHP was introduced by Brunner and coworkers

for immobilization of enzymes and antibodies[6] and for encapsulation of powdered adsorbents.[7] The agarose gel matrix offers a way of utilizing adsorbent in the most efficient powdered form for DHP. Blood corpuscles are unable to enter the interior of the gel matrix, and thus they do not come into direct contact with the adsorbent. Blood can easily flow through a column of packed enlarged beads and most of the plasma constituents including albumin can penetrate into the interior of the beads relatively easily, so even albumin-bound species, depending on the relative affinity between the adsorbent and albumin, can be removed by coming into direct contact with the adsorbent. Following the method of Brunner *et al.*, Tabak *et al.*[8] encapsulated fuller's earth in agarose beads for removal of paraquat by DHP and similarly encapsulated zirconium oxide powder for removal of inorganic phosphates. Margel[9] incorporated small polyaldehyde beads in agarose gel for preparing immunoadsorbents. They reported that the results of their *in vitro* and *in vivo* studies in rats were very promising.

For the past few years we have been directing our attention to the development of palatable dosage forms of powdered adsorbents utilizing agar.[10] In this communication we report the preparation and *in vitro* evaluation of enlarged agarose beads for use in DHP. The evaluation of the beads as a DHP column for paraquat adsorption in beagles will be reported separately.

Materials and Methods

Materials——Agarose was a product of Dojin Chemicals, designated as agarose I for electrophoresis. Kalimate (calcium polystyrene sulfonate, lot no. 1001XX) was purchased from Nikken Chemicals. Charcoal powder (Wako Pure Chemicals, lot no. PEQ 3917) was sifted and the fraction of mesh size 200—500 was employed throughout the present study. Paraquat dichloride was precipitated from Gramoxone® (Takeda Chem. Ind.) with ethanol and acetone and the precipitate was washed well with a mixture of ethanol and acetone (1 : 1). The ultraviolet-visible absorption spectrum coincided with that of an authentic sample of paraquat dichloride (purity > 99.79%) kindly supplied by ICI, Japan. The calibration curve for paraquat ions was made by using the authentic sample. All other chemicals were purchased from Wako Pure Chemicals and used as received, except for salicylic acid which was recrystallized from hot water and converted to its sodium salt. Charcoal beads developed from petroleum pitch specifically for DHP were obtained from a DHP-1 hemoperfusion cartridge (a ready-to-use column) marketed by Kuraray Co.

Preparation——Agarose (4 g) was suspended in 100 g of water and heated to 90 °C in a water bath for complete solution. Various amounts (20—30 g) of adsorbent powders were added and suspended well in the hot agarose solution. The hot suspension was drawn into a warm glass syringe and a metal needle was attached, the tip of which was cut short and perpendicular to the length of the needle. The content of the syringe was added dropwise at a rate of about 60 drops per min to a tall vessel containing an organic solvent mixture consisting of cyclohexane and chloroform (2 : 1). Only the lower part of the vessel was cooled in an ice-water bath for solidification of the beads such that the hot drops of the suspension could become spherical before solidification took place. The native beads accumulated in the vessel were frequently recovered from the organic solvent mixture and examined for deformity. Those which entrapped air were rejected. Then the beads were extensively washed with freshly distilled ether followed by water. Some native beads were crosslinked (CL) by the method of de Koning *et al.*[11] with 1,3-dichloro-2-propanol and washed with water followed by freshly distilled ether. Finally the crosslinked beads were well washed and boiled for about 30 min in water in order to check that the beads had been crosslinked and to remove the traces of organic solvents. Agarose beads without an adsorbent were similarly prepared and crosslinked. The approximate concentration of agarose in the beads without an adsorbent (control beads) was 3.8% (w/w) and that in beads containing 16% (w/w) adsorbent was 3.2% (w/w). Beads were prepared using needles of two different sizes. One was prepared as described above from a needle for lumbar puncture (o.d. 1.0 mm and i.d. 0.7 mm) and the other from an oral tube for use in rats (o.d. 1.2 mm and i.d. 0.8 mm). For the control beads and Kalimate beads containing 16% (w/w) Kalimate, the smaller needle was used. For beads containing 16% (w/w) charcoal, the larger needle was employed, since the smaller one was found to be difficult to use owing to frequent clogging. The sizes of the beads determined by measuring 20 beads each were 3.08 ± 0.07 mm for the control beads and 2.67 ± 0.12 and 2.85 ± 0.17 mm for the charcoal beads and the resin beads, respectively. Crosslinking did not alter the size of these beads. Encapsulation of Kalimate powder was possible to a level of 22% (w/w) with the larger needle. For adsorption and hemocompatibility studies, beads containing 16% (w/w) adsorbent were used. The control beads and the charcoal beads were equilibrated with Ringer's solution, and the Kalimate beads were first soaked in 10% NaCl to convert them from Ca

form to Na form and then equilibrated with Ringer's solution.

Adsorption Study——[1] Batch Experiments: All batch adsorption experiments were carried out in screw-capped test tubes (maximum capacity 15 ml) and the tubes were rotated at 37 °C end-over-end at 12 rpm for specified time periods. All experiments were performed in Ringer's solution, employing 20 mg of adsorbent powder or amounts of beads equivalent to 20 mg of the corresponding adsorbent. Kalimate powder (Ca form) was mostly converted to Na form by equilibrating it in Ringer's solution for evaluation of the effect of encapsulation into agarose beads, since the beads were employed in the experiments after equilibration with Ringer's solution. Acidic adsorbates were converted to the corresponding sodium salts by adding equivalent amounts of sodium hydroxide. At the end of the rotation periods, the concentration of adsorbate in the supernatant was assayed spectrophotometrically on a Shimadzu UV-240 double-beam spectrophotometer and the percent adsorbed was calculated. Cholate was determined by forming sulfuric acid chromogens as reported previously,[10b] and paraquat after addition of 1% $Na_2S_2O_4$ in 1 N NaOH at 600 nm. Other adsorbates were determined by direct measurement of absorbance at the wavelengths of maximum absorbance.

[2] *In Vitro* Hemoperfusion: An adsorbate was added to 100—120 ml of freshly obtained heparinized rabbit blood (100 U/ml of blood). A bed of 15 ml of beads packed in an Amicon column GF16 × 15 was continuously perfused from bottom to top with the blood at a rate of 3 ml/min (unless otherwise stated) using a tubing pump (Taiyo, Decarf-N, type N-10) and at 30 ml/min using a Masterflux mode 7015.12 (Cole-Parmer Instrument Co.). Tygon tubing was employed as the blood line and the perfusion was carried out at room temperature for 90 min to 5.5 h. Plasma levels of various additives were determined as follows: theophylline by high-performance liquid chromatography; urate and cholate by the enzymatic methods; and paraquat by extraction employing an ion-exchange resin followed by colorimetric assay as described above using 10 cm cells.

Blood Compatibility Test——A batch experiment employing plastic centrifuge tubes were carried out for all 3 types of agarose beads, using both crosslinked and uncrosslinked (native) beads of each type. One gram of each kind of wet beads (Ringer's solution was blotted from the surface) was weighed and placed in the tube together with 9 ml of freshly obtained heparinized (100 U/ml) rabbit blood. The tubes were rotated at 12 rpm, at 37 °C for 90 min. The control tube containing a mixture of 9 ml of the blood and 1 ml of Ringer's solution was subjected to the same treatment as the tubes containing beads. Erythrocytes and leucocytes were counted on a Toa blood cell counter (model CC 108) and platelets were counted by the ordinary smear staining method.[12] Some plasma components were analyzed for possible adsorption on these beads by the usual clinical laboratory methods.

Results and Discussion

Preparation of the Beads

The main difference between the present method and that of Brunner *et al.*[7] lies in the selection of the organic solidifying solvent system employed. Their system consisted of toluene, chloroform, and hexane (5:2:1), the main component being toluene which is strongly adsorbed by charcoal. Of the many solvent systems investigated, the system consisting of cyclohexane and chloroform (2:1) was found to be satisfactory for all the beads prepared.

Encapsulation of charcoal was more difficult than that of Kalimate and the larger needle was required. Beads containing 22% (w/w) Kalimate were just as easily prepared with the

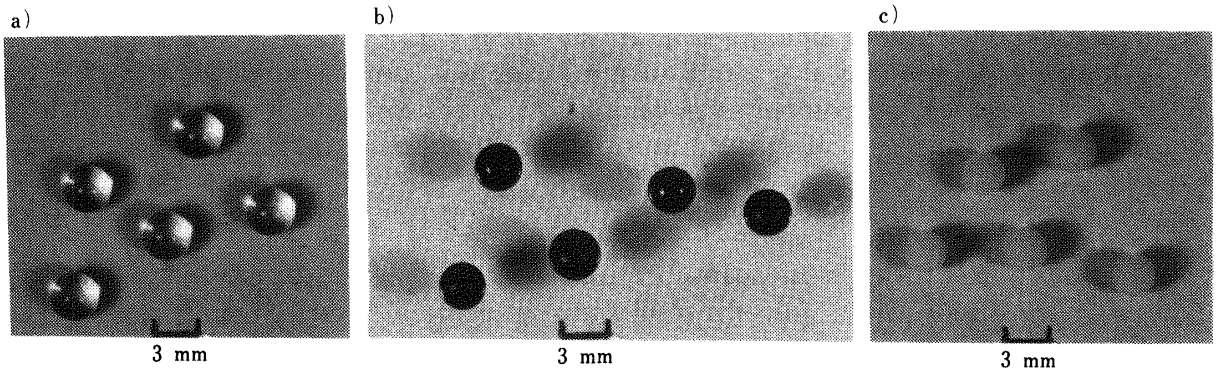

Fig. 1. Three Types of Agarose Beads

a) Control agarose beads without an adsorbent. b) Agarose-encapsulated charcoal beads. c) Agarose-encapsulated cation-exchange resin beads.

TABLE I. Effect of Encapsulation of Charcoal Powder in Agarose Beads on Adsorption

Adsorbate	Time (h)	% adsorbed[a] Powder	% adsorbed[a] Beads (CL)[b]
Paraquat	1	22.9±5.3	22.1±3.2
	3	31.1±2.0	28.3±1.5
	24	31.9±3.3	31.0±2.0
Salicylate	1	45.4±1.4	17.3±2.3
	3	44.6±0.8	28.5±1.7
	24	46.9±2.8	34.5±0.9
Cholate	1	97.1±1.5	22.5±2.6
	3	95.7±0.1	34.3±3.0
	24	95.4±0.2	68.2±1.3

a) The percentage of adsorbate adsorbed (mean±S.D. of 2—6 determinations) by an amount of adsorbent equivalent to 20 mg of activated charcoal powder (mesh 200—500, dried at 110 °C) when shaken at 12 rpm and 37 °C with 2 mM adsorbate in Ringer's solution (total volume 10 ml). *b*) Crosslinked beads containing 16% (w/w) charcoal.

TABLE II. Effect of Encapsulation of the Cation-Exchange Resin into Agarose Beads on Paraquat Adsorption

Time (h)	% of paraquat adsorbed[a] Powder	% of paraquat adsorbed[a] Beads (CL)[b]
1	63.5±2.1	36.3±1.1
3	66.5±1.4	54.8±1.1
24	67.0±0.0	70.1±0.9

a) The percentage of paraquat adsorbed (mean±S.D. of 2—6 determinations) by an amount of adsorbent equivalent to 20 mg of the cation-exchange resin powder when shaken at 12 rpm and 37 °C with 2 mM paraquat in Ringer's solution (total volume 10 ml). *b*) Crosslinked beads containing 16% (w/w) resin.

TABLE III. Effect of Crosslinking (CL) on the Adsorption by Three Types of Agarose Beads

Adsorbate	Time (h)	% adsorbed[a] Control beads[b] Native[c]	Control beads[b] CL	Charcoal beads Native[c]	Charcoal beads CL	Resin beads Native[c]	Resin beads CL
Theophylline[d]	1	2.8	0	86.8	86.4	3.9	5.8
Cholate[d]	1	2.1	0	65.7	65.7	0	0
	24	0	2.6	99.1	97.6	0	0
Paraquat[e]	1	4.8	3.6	61.6	64.8	91.3	92.9
	24	11.3	8.8	77.3	83.3	98.0	98.1

a) Beads (1.0 g) were shaken at 12 rpm and 37 °C in 9.0 ml of Ringer's solution containing an adsorbate. *b*) Agarose beads without an adsorbent. *c*) Native means uncrosslinked beads. *d*) Total adsorbate added was 90 μmol. *e*) Total adsorbate added was 54 μmol.

larger needle as those containing 16% (w/w) with the smaller needle.

Photographs of the three different types of crosslinked beads, two with 16% (w/w) adsorbent and one without an adsorbent, are shown in Fig. 1.

Although the mechanical strength of our beads was not measured, crosslinking was reported[11] to increase the compressive strength by a factor of 4. We observed no rupture of our crosslinked beads on boiling them in water for 30 min or in the courses of subsequent experiments.

In Vitro Adsorption Study

The effect on adsorption of encapsulation of charcoal in agarose beads was studied for 3 different types of adsorbates, and the results are presented in Table I. The results with the resin in agarose beads for paraquat adsorption are shown in Table II. With all adsorbates the rates of adsorption were reduced for the beads. For paraquat adsorption, the capacity of the charcoal powder (Table I) and that of the resin (Table II) were unchanged upon encapsulation into agarose gel, with the resin beads being more efficient than the charcoal beads. From the

TABLE IV. Comparison between Adsorbent Containing Agarose Beads (CL) and Kuraray Beads (DHP-1) for Adsorption of Paraquat and Cholate

Time (h)	% adsorbed[a]			
	Paraquat		Cholate	
	Agarose (resin)	DHP-1	Agarose (charcoal)	DHP-1
1	36.3±1.1	17.5±0.0	22.5±2.6	2.2±4.8
3	54.8±1.1	21.5±1.4	34.3±3.0	11.8±5.8
24	70.1±0.9	23.1±0.9	68.2±1.3	20.7±2.9

a) When an amount of beads equivalent to 20 mg of adsorbent powder was shaken at 12 rpm and 37 °C with 2 mM adsorbate in Ringer's solution (total volume 10 ml), mean±S.D. (2—6 determinations).

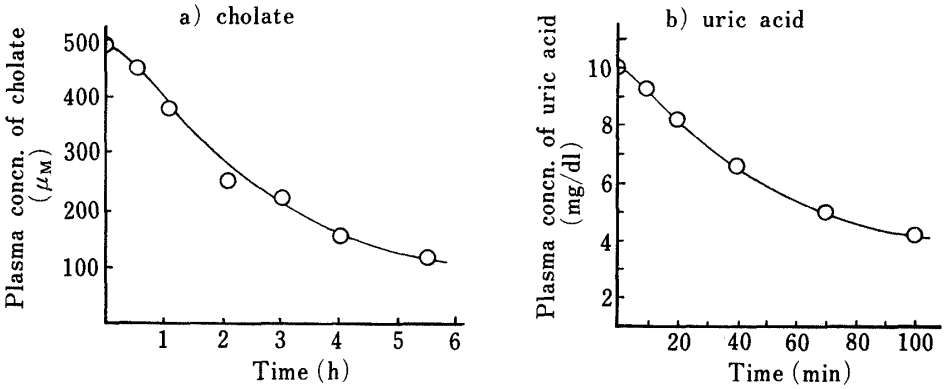

Fig. 2. Elimination by Adsorption from Rabbit Blood Studied by the Column Method

Fresh heparinized (100 U/ml) rabbit blood (100 ml) was circulated at 3 ml/min through a column containing 9.3 g of charcoal beads (equivalent to 1.5 g of charcoal) at about 32 °C.

increase in the amount of adsorption with time, the capacity probably remains unchanged for salicylate and cholate as well. The finding that crosslinking does not affect the adsorption behavior of the beads (Table III) supports this view. The reduction in the rate of adsorption for the adsorbents in agarose beads in comparison with the naked adsorbents is dependent upon the nature of the adsorbate. Thus, it may depend upon the permeability of the adsorbate in the gel, factors such as the size, electric charge, shape, *etc.* being responsible. Paraquat (M_r 186) with two positive charges probably diffuses more easily in the hydrophilic gel than other adsorbates. The smallest rate, shown by cholate, is likely to be attributable mainly to the large size (M_r 408).

Paraquat adsorption was compared between the resin beads (CL) and DHP-1 beads, and cholate adsorption between the charcoal beads (CL) and DHP-1 beads. The results (Table IV) indicate that agarose-encapsulated beads are approximately 3 times more efficient than DHP-1 beads at both 3 and 24 h. Our previous study[10f)] indicated that the petroleum-pitch based carbon in the bead form adsorbed paraquat just as effectively as when it was pulverized. However, cholate was very poorly adsorbed by the original beads, the efficiency being greatly increased by pulverization to the level of the charcoal presently employed for agarose beads. Adsorption of paraquat by both beads is unlikely to be diffusion controlled. Thus, the better adsorption of paraquat by the agarose beads than by DHP-1 should be due to the greater capacity of the resin itself. On the other hand, the less efficient adsorption of cholate by DHP-1 is due to the reduced rate of adsorption and not to lower capacity of the beads. The larger cholate ions are less able to penetrate into the interior of DHP-1 beads which are membrane-

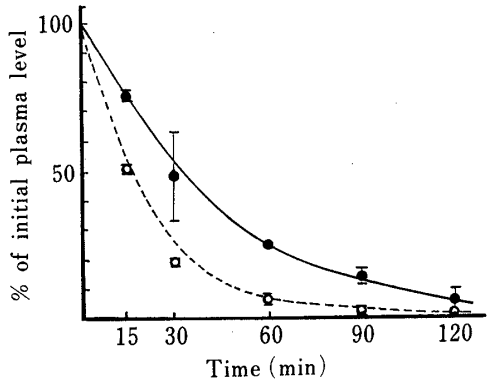

Fig. 3. Effect of Rate of Circulation on the Elimination of Theophylline from Rabbit Blood by Adsorption

—●—, 3 ml/min; ---○---, 30 ml/min.
Details as for Fig. 2.
Initial theophylline concentrations were 17.2 and 36.7 μg/ml.
The vertical bar represents the mean ± S.D. ($n=2$).

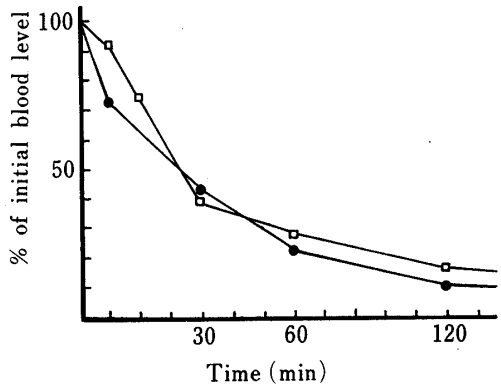

Fig. 4. Elimination of Paraquat by Adsorption, Studied by the Column Method

To 120 ml of fresh heparinized (100 U/ml) rabbit blood, 6.4 mg paraquat dichloride was added. The blood was circulated at 3 ml/min through a column containing 9.3 g of beads (equivalent to 1.5 g of adsorbent) at about 32 °C.
—□—, agarose-encapsulated cation-exchange resin beads.
—●—, agarose-encapsulated charcoal beads.

coated solid charcoal beads with diameters ranging from 0.4 to 1.0 mm. In hemoperfusion, the rate of adsorption is an important factor. Thus, the high surface area of the powdered form of adsorbents and the low resistance to the diffusion of adsorbates in a porous agarose gel matrix offer a particularly desirable combination for increasing the rate of adsorption of relatively large adsorbates such as cholate.

Adsorption profiles from blood by the charcoal columns (uncrosslinked beads) are shown in Fig. 2 for cholate and urate, and in Fig. 3 for theophylline. The adsorption efficiency of the columns is in parallel with the results of the *in vitro* adsorption study in Ringer's solution shown in Table I. The higher rate of flow is more efficient as shown for theophylline adsorption. For paraquat adsorption, two types of beads are compared in Fig. 4. The results show that the resin beads are more efficient than the charcoal beads for adsorption from blood, as from Ringer's solution.

Hemocompatibility

Table V summarizes the hematological changes after 90 min of contact between the blood and various beads. In all cases crosslinking was considered unlikely to influence blood compatibility. Although reduction in the erythrocyte and leucocyte counts was not significant, the control agarose beads reduced platelets by about 25%. A similar level of reduction in platelet counts was observed for the charcoal beads. With the resin beads, about 35% reduction was observed.

The results of biochemical tests on certain selected blood constituents for only the crosslinked beads are shown in Table VI, since no significant difference was observed before and after crosslinking for all the constituents assayed. Slight hemolysis must have occured for all types of agarose beads, as shown by the higher lactate dehydrogenase level as compared with the control blood. Charcoal is expected to adsorb creatinine, uric acid, calcium, free fatty acids, bilirubin, and glucose. The slight reductions in these values for the charcoal beads as compared with the control agarose beads may reflect this tendency. Although charcoal is expected to adsorb well the lipid components, these are not (or are only marginably) removed from the blood by the charcoal beads since they are present as lipoproteins. Because of the equilibration of the resin beads prior to the study, only slightly higher values were observed for K^+ and Ca^{2+} than in the case of the control agarose beads.

TABLE V. Effects of Various Agarose Beads on the Counts of Blood Corpuscles; Batch Experiment[a]

Count/μl	Control blood	Control beads[b]		Charcoal beads		Resin beads	
		Native	CL	Native	CL	Native	CL
Erythrocytes							
$\times 10^{-4}$	541	538	544	546	540	520	530
(%)	(100)	(99)	(101)	(101)	(100)	(96)	(98)
Leucocytes							
	5700	5700	5900	5400	5300	5700	5700
(%)	(100)	(100)	(104)	(95)	(93)	(100)	(100)
Platelets							
$\times 10^{-4}$	38.4	28.3	31.6	30.4	28.7	24.8	24.5
(%)	(100)	(74)	(82)	(79)	(75)	(65)	(64)

a) Fresh heparinized (100 U/ml) rabbit blood (9 ml) was added to 1 g of beads, either crosslinked (CL) or uncrosslinked (native), in a plastic centrifuge tube and the tube was rotated at 12 rpm and 37 °C for 90 min. *b*) Agarose beads without an adsorbent.

TABLE VI. Effects of Various Agarose Beads on the Levels of Some Plasma Constituents at 37 °C; Batch Experiments[a]

Plasma constituent	Blood control[b]	Agarose beads (CL)		
		Control[c]	Charcoal	Resin
Total protein (g/dl)	5.3	5.8	5.5	5.6
Albumin (g/dl)	2.0	2.1	2.0	2.0
Lactate dehydrogenase (WU)	247	407	328	427
Total cholesterol (mg/dl)	63	73	69	69
Triglyceride (mg/dl)	72	83	76	76
β-Lipoprotein (mg/dl)	37	25	33	33
Free fatty acid (meq/l)	2.25	2.21	2.08	2.21
Total bilirubin (mg/dl)	0.3	0.2	0.1	0.3
Urea nitrogen (mg/dl)	21.1	20.3	20.5	20.3
Creatinine (mg/dl)	0.8	0.8	0.3	0.6
Uric acid (mg/dl)	1.7	2.3	1.9	2.3
Na^+ (meq/l)	146	147	145	146
K^+ (meq/l)	3.7	3.5	3.7	4.0
Ca^{2+} (meq/l)	12.8	13.0	12.0	14.5
Cl^- (meq/l)	117	109	109	109
Glucose (mg/dl)[d]	70	69	67	69

a) Fresh heparinized (100 U/ml) rabbit blood (9.0 ml) was added to 1.0 g of beads in a plastic centrifuge tube and the tube was rotated at 12 rpm at 37 °C for 90 min. The beads had previously been equilibrated with Ringer's solution. *b*) The blood (9.0 ml) and 1.0 ml of Ringer's solution were placed in the tube and the tube was similarly rotated. *c*) Agarose beads without an adsorbent. *d*) NaF was not added.

Conclusion

The use of agarose for encapsulation of an adsorbent powder, proposed by Brunner, was shown to be promising. The adsorption capacity of the beads is retained but the rate may be reduced, depending upon the substance to be removed. Although charcoal has a wide spectrum of adsorption, ion-exchange resins may be more efficient for ionic substances. The crosslinked beads can be heat-sterilized and no serious blood incompatibility problems can be foreseen, at least in short-term use.

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