PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 1114–1118 (1974)

The Binding of Bilirubin to Agar[1] (38256)

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(Introduced by H. C. Harrison)

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While studying bacterial degradation of bilirubin, it was found that agar protected bilirubin from bacterial action by adsorption of the bilirubin (1). In preliminary animal experiments, variation was found between batches of agar in its effect on fecal excretion of bile pigments and on lowering of serum bilirubin levels in the Gunn strain of rat. The binding of bilirubin to agar was studied in part to explain the variation seen in the animal studies.

Agar is a seaweed extract which can be fractionated into agarose and agaropectin. Agarose is a linear polymer composed of alternating units of D-galactose and 3,6-anhydro-L-galactose (2). Agaropectin has the same backbone as agarose but in addition, some of the hydroxyl groups are esterified with sulfuric or glucuronic acid (3). The insolubility of agar in water at temperatures below 90° provides an opportunity to study the binding of bilirubin to agar by taking advantage of a solid-liquid interface formed by the suspension of agar powder in aqueous solutions of bilirubin. By varying the bilirubin concentration, the binding of bilirubin by agar could be analyzed by the adsorption isotherm technique as described by Klotz (4). The equation:

$$\frac{1}{\bar{\nu}} = \frac{1}{nK_a\,(\mathrm{A})} + \frac{1}{n}$$

is derived from the law of mass action where $\bar{\nu}$ represents the number of moles of bound complex per mole of large molecule (agar); n the number of binding sites per mole of agar; (A) the molar concentration of free bilirubin; and K_a the association constant. Assuming equal and noninteracting binding sites, the plot of $1/\bar{\nu}$ versus $1/(\mathrm{A})$ will be linear and will give values for n and K_a derived from the Y intercept and the slope respectively. In representing these data, a mole of agar has been arbitrarily designated 5×10^4 g.

Materials and Methods. The U. S. P. agar powder used in these experiments was obtained from the Amend Drug Company (lot #604066) unless otherwise specified. Bilirubin was purchased from Pfansthiel Laboratories (lot #9389-L) and had a molar extinction in chloroform of 58.5×10^3 at 454 nm in our laboratory. The water was triple distilled and deaminated with a conductance of 0.6–1.0 micromhos. Bilirubin glucuronide was extracted from human gallbladder bile and purified by the method cited by Jacobsen (5). Other chemicals were obtained from J. T. Baker.

All experiments with bilirubin were done under dim lighting to avoid its photo-oxidation. The stock solutions of bilirubin were freshly prepared for each experiment by dissolving crystalline bilirubin in deaerated 0.1 *M* sodium hydroxide to a concentration of about 3.4 m*M*. The concentrations of the bilirubin added to the incubation mixtures were checked by the extinction at 454 nm of an aliquot extracted into chloroform.

[1] Supported by United States Public Health Service Grants Nos. HD 02268 and HD 00091.

The assays were done in a series of 16 × 100 mm screw cap test tubes with 5 mg of agar per tube. To each tube, 2 ml of solution was added containing 0.14 *M* sodium chloride, 0.02 *M* sodium phosphate buffer (pH 7.40), 0.001 *M* sodium sulfite and 0.017 to 1.4 m*M* bilirubin. The inorganic salts were added in 1 ml and the second milliliter contained the bilirubin stock solution diluted with varying amounts of 5 m*M* sodium hydroxide solution. The final pH in each tube was 7.91 ± 0.09. The tubes were mixed on a Vortex shaker and rotated in a near horizontal position at 10 rpm on a multipurpose rotator[2] in a 37° room for 90 min.

The mixtures were then centrifuged at 1500 *g* for 10 min and extinction at 440 nm of the supernatants (unbound fractions) was read on a Zeiss PMQII spectrophotometer. Aliquots were diluted if the optical density exceeded 0.5 units. These aliquots were then returned to their tubes and recoveries were obtained by adding 8 ml of chloroform to each tube along with 0.5 ml of 0.1 *M* sodium EDTA, pH 6.0. The tubes were mixed well and stored overnight at 4° in the dark. Then two drops of 4 *N* hydrochloric acid were added, mixed and the suspensions stored overnight at 4° in the dark. The solvents after remixing were separated by centrifugation and the optical density of the chloroform layer was measured at 454 nm. These data were used to plot an adsorption isotherm and replotted according to the method of Klotz (4).

The time for equilibrium was determined with two initial bilirubin concentrations distributed into 2-ml aliquots and removed from the 37° incubator and centrifuged at selected intervals. A series of adsorption isotherms were done in which pH, ionic strength (sodium chloride concentration) and temperature were varied singly. The influence of divalent cations was studied as follows: Two agar samples (500 mg each) were washed three times with 30 ml of either (a) 0.1 *M* sodium ethylenediaminetetra-acetate (EDTA), pH 7.5 or (b) distilled water with 15–20 min of gentle agitation between washes. The EDTA-washed sample (a) was then washed with distilled water several times. The supernatants were saved and assayed for calcium using a Perkin-Elmer atomic adsorption spectrometer (kindly provided by Dr. Helen Harrison) and for EDTA by the titration method of Harrison and Harrison (6) using eriochrome black dye. These samples were then lyophilized and weighed aliquots were hydrolyzed in 6 *N* hydrochloric acid and assayed for calcium content. The samples were also assayed for bilirubin binding capacity using tris buffer instead of phosphate with and without the addition of calcium or magnesium. The sulfate content of the agar was determined by digestion in 6 *N* hydrochloric acid for 4 hr at 100°. Sulfate was precipitated with barium and concentrations estimated by the turbidimetric method of Dodgson and Price (7). The binding of other organic ions by agar was studied. The compounds studied were sulfanilate, salicylate, HABA dye,[3] BSP dye and conjugated bilirubin. These compounds were substituted for bilirubin in the

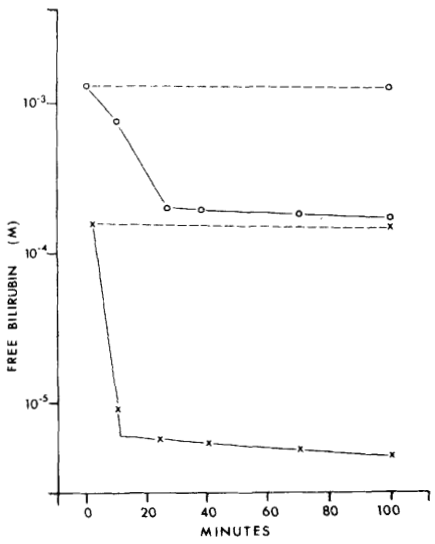

FIG. 1. Time course. Starting concentrations of 1.2 m*M* and 0.15 m*M* are represented. Solid lines represent tubes with 5 mg of agar. Dotted lines represent tubes without agar under otherwise standard conditions.

[2] Scientific Industries, Springfield, MA.

[3] Dade.

assay and measured by standard clinical laboratory methods. The binding of bilirubin by other polysaccharides (cellulose, phosphocellulose and DEAE cellulose) was studied by their substitution for agar in the isotherm assay. The effect of urea on the binding of bilirubin to agar was also evaluated.

Results. The time course of the binding of bilirubin by agar is shown in Fig. 1. Without agar, both high (1.2 mM) and low (0.15 mM) bilirubin concentrations decayed slightly over a 100-min period. With agar, a rapid fall of bilirubin concentration in the supernatant occurred over the first 25 min. With the higher concentration of bilirubin, the equilibrium was reached later. After 25 min, the supernatant bilirubin concentration showed a small amount of decay in all tubes. Figure 2 shows a typical adsorption isotherm. There was a consistent sigmoid shape of the curve beginning at low concentrations of bilirubin followed by a rectangular hyperbola. The replot of the hyperbolic portions (14 points) revealed 25.6 moles of equal and noninteracting binding sites per 50,000 g of agar with an association constant (K_a) of 2.35×10^5 (Fig. 2b). Recoveries of bilirubin averaged 94% of additions for all experiments.

The amount of bilirubin bound increased with the decrease in pH. At ionic strengths higher than 0.5, bilirubin precipitated in the absence of agar. At ionic strengths lower than 0.5, the number of binding sites increased with decreasing ionic strength. In an Arrhenius plot with temperatures vary-

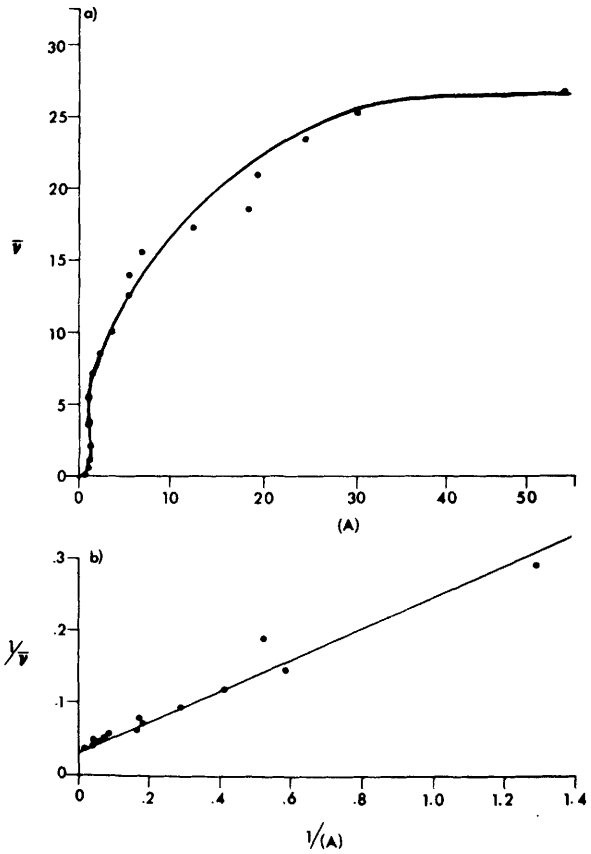

FIG. 2a. Adsorption isotherm. $\bar{\nu}$ represents moles of bilirubin bound per 50,000 g of agar. (A) represents the concentration of bilirubin left in the supernatant (μM).

FIG. 2b. Inverse plot of the above data according to the method of Klotz.

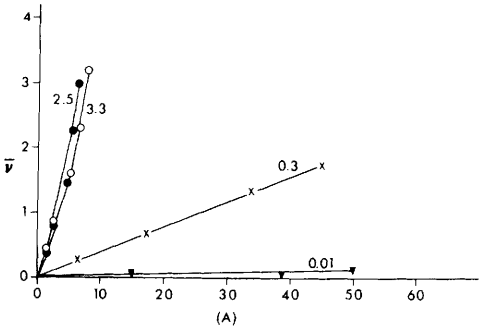

FIG. 3. Calcium requirements. $\bar{\nu}$ and (A) are defined as in Fig. 2a. Numbers adjacent to curves represent moles of calcium present per 50,000 g of agar. Open circles represent untreated agar. Triangles represent decalcified agar. Closed circles and ×'s represent decalcified agar with readdition of calcium to incubation mixture.

ing from 0° to 50°, there was an increase in apparent association constant with a rise in temperature. The Gibbs free energy appeared to be about 2 kcal/mole. The binding of bilirubin to EDTA-treated agar is shown in Fig. 3. Less than 0.2 moles of the EDTA per 50,000 g of agar remained after washing of the agar. The calcium-free agar showed very little affinity for bilirubin. The binding of bilirubin increased after the addition of calcium to calcium-depleted agar and returned to the values of untreated agar (Fig. 3). Equimolar amounts of magnesium had much less of an effect.

The binding capacities of a variety of agars and of agarose and agaropectin are shown in Table I. These products are also compared in relationship to sulfate and calcium contents in moles per 50,000 g of agar. There was variation in the association constant between batches that was no greater than the variation between assays of the same batch. The determination of the number of binding sites appeared to be more reproducible. In general, the number of binding sites (n) increased with the sulfate content (rank correlation coefficient $\{r_s\}$ of 0.673, $P < 0.02$) and with the calcium content ($r_s = 0.554$, $P < 0.05$) (8).

None of the other organic ions studied had a measurable affinity for agar and agar had a greater affinity for bilirubin than cellulose, DEAE-cellulose and phosphocellulose. When 3.2 M urea was added to the incubation mixture, the apparent number of binding sites on agar was reduced without significant reduction in association constant. Higher concentrations of urea precipitated bilirubin in the absence of agar.

Discussion. We do not know how bilirubin binds to agar. The consistent sigmoid

TABLE I. Properties of Various Agar Products.[b]

Source	Lot #	Association constant (K_a) × 10^5	Binding sites[a] (n)	Sulfate[a]	Calcium[a]
Seravac (Agarose)	179	1.53	25.3	0.03	0.9
Amend	C604066	1.54	25.5	2.7	3.1
MacDowell	4037	1.14	27.0	3.9	4.1
MacDowell	4278	1.34	27.3	10.7	3.8
Amend	C710542	1.17	31.3	6.8	8.7
Seravac (Agaropectin)	—	2.74	32.3	1.3	2.4
Sigma	109B0290	2.92	32.8	9.0	9.1
Sigma	20C0880	3.98	36.8	9.8	8.3
Fisher	7717941	2.22	48.8	9.1	7.6
Penick	125LLP011	1.88	50.1	8.7	6.2
Fisher	766501	0.51	55.6	10.9	5.7

[a] Moles/50,000 g of agar.

[b] There was a positive correlation (rank correlation coefficient, $r_s = 0.673$, $P < 0.02$) between n and the sulfate content, another between n and the calcium content ($r_s = 0.554$, $P < 0.05$) and also between the calcium and sulfate content ($r_s = 0.527$, $P < 0.06$).

shape of the isotherms suggests cooperativity but might also be explained by an error in measuring very small concentrations of bilirubin. The two end-pyrrole nitrogens of bilirubin and the hydroxyl groups of agar are available for hydrogen bonding. The effects of urea and of temperature are consistent with hydrogen bond formation. It is also conceivable that the cross-linked agar strands provide relatively apolar interstices for the trapping of hydrophobic bilirubin. The effects of pH and ionic strength on binding and the lack of binding of more polar organic ions are compatible with hydrophobic interaction. Further work is necessary to define the actual mechanism.

The data suggest that bilirubin binds specifically to agar and that the measured association constant for the reaction is approximately 10^5 in all the agar products tested. The amount of bilirubin bound is roughly related to the calcium and sulfate content. However, since the number of bilirubin binding sites exceeds the sulfate and calcium content per 50,000 g of agar, calcium and sulfate are unlikely to be part of the binding site itself. Cross-links between carbohydrate chains can be formed by a calcium ion binding to two sulfate ions. This configuration has been described in carrageenans which are structurally similar to agar (9). The cross-links between chains would then render agar able to bind bilirubin. When half the sulfate content or the calcium content is considered, the smaller of the two values would then be the limiting factor for the formation of binding sites. When this value was ranked along with n for each product, there was a strong correlation ($r_s = 0.836$, $P < 0.001$). The variation in bilirubin binding between agar batches that was noted in animal experiments seems now to be related to the calcium and sulfate content.

Summary. The binding affinity of agar for bilirubin was measured *in vitro* by suspending agar in isotonic solutions of pH 7.9 containing concentrations of bilirubin between 0.017 to 1.4 m*M*. The affinity constant for bilirubin was 10^5 and the number of binding sites (n = 0-55/5.0 × 10^4 g of agar) was dependent upon the calcium and sulfate contents of the agar. Calcium depleted agar had no affinity for bilirubin but regained its affinity by addition of calcium proportionate to the sulfate content. The number of moles of bilirubin bound by agar greatly exceeded the molar content of Ca^{2+} and SO_4^- and suggested that bilirubin was bound between the hydrophobic interstices of the cross-linked strands of agar. Water soluble organic anions did not show significant binding to agar.

We are grateful to R. L. Williams and E. L. Leffler for technical assistance, to V. O'Rourke and P. Staeheli for their help in preparing the manuscript, and to Dr. Jan Cejka for his critical review of the manuscript.

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Received Feb. 19, 1974. P.S.E.B.M., 1974, Vol. 146.