Rate Studies on the Binding of Bilirubin by Ion-Exchange Resins

By R. J. SAWCHUK and J. G. NAIRN

The rate of binding of bilirubin by strongly basic anion-exchange resins was determined by the batch method. The rate of uptake of bilirubin from solution was found to follow a second-order rate expression, and the rate constant was dependent upon the size of the resin beads and the degree of crosslinking of the resin. It was concluded that the rate of binding, which was influenced by the fractional pore volume of the resin phase, was controlled mainly by a particle diffusion mechanism. Aqueous alkaline solutions of bilirubin of improved stability were prepared in order to study the binding phenomena.

A FEW CASES have been reported in which abnormally high serum levels of bilirubin in humans and animals have been decreased by the oral administration of an ion-exchange resin. Lester *et al.* (1) having investigated both the absorption and transport of unconjugated bilirubin across the intestinal mucosa, made the following postulation, "If it were possible to achieve irreversible binding of intestinal bilirubin, its reabsorption would be prevented, the efficiency of pigment disposition would be increased, and the plasma level (and, consequently, the level in the total miscible pool) would be reduced to a new, lower plateau." They demonstrated that high serum levels of bilirubin in rats, due to administration of labeled bilirubin, could be effectively reduced by adding an ion-exchange resin to the rat's diet. A report (2) of a case of xanthomatous biliary cirrhosis in a patient showing a slowly progressive jaundice described the reduction of bilirubin levels after administration of an ion-exchange resin. A reduction in serum levels of bilirubin has been obtained using an ion-exchange resin in a case of intrahepatic biliary atresia (3). A strongly basic quaternary ammonium ion-exchange resin has also been used to bind bile acids (4, 5).

Some work has been published on the *in vitro* uptake of bilirubin by ion-exchange resins. The pigment has been removed from solutions by a resin in a column (3). The transfer of bilirubin from albumin to resin has also been achieved (6). Other papers (7, 8) describe the problems of eluting bilirubin from ion-exchange resins. Preliminary work for the present research involved several attempts to elute bilirubin from ion-exchange resins, which had previously been in contact with a solution of bilirubin. These attempts included both column and batch methods. Solvents such as dioxane, tetrahydrofuran, and aqueous solutions of sodium hydroxide, sodium acetate, or hydrochloric acid were employed; however, satisfactory removal of bilirubin from ion-exchange resins was not achieved.

The purpose of this research was to investigate the rate of binding of bilirubin to ion-exchange resins *in vitro*. The rate of binding of bilirubin as a function of crosslinking and particle size of the resin was studied in detail so that properties of the resin which control the rate could be elucidated. This was achieved by allowing solutions of bilirubin to come in contact with resin beads using the batch technique.

The lack of stability of bilirubin in both organic and aqueous solvents has posed a serious problem to researchers and clinicians. Several factors, such as light, oxygen, pH, metals, solvent, and temperature cause decomposition (9–14). The kinetics of decomposition have also been reported (15, 16). Many agents and techniques have been employed to stabilize solutions of bilirubin, for example: serum (14, 17), temperature (18), chemicals (19–21), a complexing agent (14), and nitrogen (22). Unfortunately, as the solubility of bilirubin increases with pH (23) so also does the rate of decomposition (16). In this study it was desirable to minimize the decomposition of bilirubin in solution. This was achieved after considerable preliminary investigation by using a weak base to achieve a suitable pH for solubility, employing a nitrogen atmosphere to reduce oxidation, maintaining the reaction vessel in the dark, and carrying out the experiments at a low temperature.

Another serious problem which had to be solved was the deposition of bilirubin from solution onto all surfaces except a clean smooth glass surface,

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TABLE I—SUMMARY OF DATA OBTAINED FOR THE BINDING OF BILIRUBIN TO ION-EXCHANGE RESINS

Resin	Mesh Size	Average Wet Bead Radius, r, mm.[a]	Capacity, b, meq./g. dry resin[b]	Moisture Content, % w/w[b,c]	Rate Constant, k, l./(meq. hr.)[b]
1-X1	50–100	0.213	4.72	86.8	0.99
1-X2	50–100	0.179	4.42	72.0	0.65
1-X4	20–50	0.285	4.21	52.8	0.061
1-X4	50–100	0.151	4.20	53.8	0.19
1-X4	100–200	0.076	4.14	64.8	2.4
1-X4	200–400	0.038	4.08	61.3	2.2
2-X4	20–50	0.285	4.02	47.7	0.029
1-X8	50–100	0.137	3.77	45.5	0.045
2-X8	50–100	0.137	3.58	41.0	0.054

[a] The average wet bead radius was determined from data in *Reference 36*. [b] The values given are the average of duplicate determinations. [c] Fractional pore volume (ϵ) = % moisture content/100.

and consequently a special stirring apparatus was used (24).

EXPERIMENTAL

Materials—The strongly basic resins[1] were washed, and conditioned by conversion to the hydroxide, the chloride, and back to the hydroxide form. The resin was then converted to the chloride form and washed thoroughly with freshly boiled, deionized water and stored under deionized water. Samples of the conditioned resin for experiments and characterization were centrifuged at 1,300 r.p.m. for 30 min. in a sintered-glass filter tube of medium porosity to remove interstitial water. The moisture contents of the resins were determined by heating the resin in an oven at 110° for 12 hr. The scientific or equivalent weight capacities of the resins were determined by converting the resin to the hydroxide form by the column method, rinsing the resin with boiled deionized water, eluting the hydroxide with sodium chloride solution, and titrating the effluent with hydrochloric acid. The results of these determinations are given in Table I. The bilirubin used was Fisher Certified reagent grade, lot No. 740759. The molar absorptivity was within the range of proposed standards for pure bilirubin (25). IR analysis of bilirubin by the potassium bromide disk method agreed with the literature (22).

Reaction Mixtures—A blank for spectrophotometric analyses and a suitable solvent for bilirubin was prepared by adding 1.2 ml. of a 5% v/v aqueous solution of freshly distilled *n*-propylamine to 650 ml. of boiled, deionized water which had been previously treated by bubbling with high purity nitrogen for 45 min. Bilirubin, approximately 4 mg., was added in the dark to 580 ml. of the above solvent. The mixture, kept under a nitrogen atmosphere, was protected from light and shaken at room temperature for 1.5 hr. and at 3° for 0.5 hr. in order to effect solution.

An accurately weighed quantity of centrifuged resin in the chloride form, equivalent to approximately 12 mg. of dry resin was added to a conical flask onto the shoulder of which was fused a square spectrophotometric cell. The flask was covered with aluminum foil and was equipped with a stirring apparatus described previously (24). A second flask was prepared in the same manner for the duplicate experiment. A third flask without resin was used as a control. The cold bilirubin solution (190 ml.) was added to each flask in the dark, nitrogen was swept over the surface, and the flasks were stoppered.

Analyses—The percentage transmittance of the bilirubin solutions was determined (and converted to absorbance values) at 432 mμ, the wavelength of maximum absorbance, on a Bausch and Lomb Spectronic 20 spectrophotometer fitted with a square-cell adapter. The concentration of bilirubin remaining in solution was determined from a Beer-Lambert plot of bilirubin in the water–*n*-propylamine solvent.

Kinetic Studies—Spectrophotometric analyses were carried out as soon as practical, and the solution in the cell was returned to the body of the flask. The flasks were positioned in a water bath (5.3 ± 2.0°), over magnetic mixers, which had been adjusted to 360 r.p.m. The experiment was protected from light at all times. Analyses of the mixtures were made at suitable intervals, by removing the flasks from the bath, allowing the resin beads to settle, and then pouring some of the solution into the attached cell.

Ion-exchange resin beads of 100–200 and 200–400 mesh sizes were found to adhere to the flask wall, and occasionally when they entered the spectrophotometric cell, they could not be returned to the flask after the analysis had been completed. This problem of beads adhering to the glass was solved by coating the inside of the vessels with Dow Corning 200 Fluid (350 CS.) using the method recommended by the manufacturer, thereby effecting agitation of all beads by the magnetic stirrer.

RESULTS AND DISCUSSION

The capacities and moisture contents which were determined in this study are given in Table I.

The stability of the bilirubin in solution was determined by noting the change in the absorbance in the control flasks. The maximum change of absorbance in the control flasks for the data presented in Fig. 1 (*i.e.*, for the first 14 hr.) was never greater than 1%. A few of the solutions were observed for longer periods of time (about 50 hr.) when the rate of binding was very low, and in some of these experiments the change of absorbance was as high as 2.6%.

Since the binding of ions to a resin is a diffusion phenomenon (26) the rates of binding can best be compared by evaluating diffusion coefficients, which

[1] Dowex 1 and Dowex 2, Dow Chemical Co., Midland, Mich.

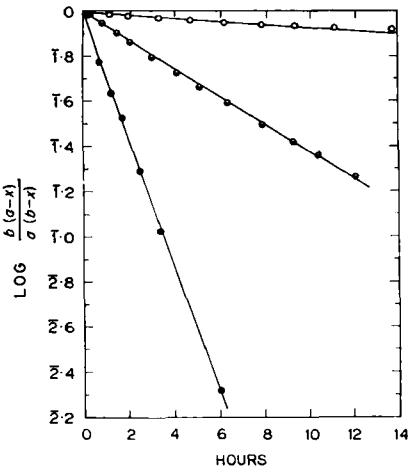

Fig. 1—Plots illustrating the removal of bilirubin from solution by ion-exchange resins. Key: ○, *1-X4 (20–50 mesh);* ◒, *1-X2 (50–100 mesh);* ●, *1-X4 (200–400 mesh).*

are obtained by considering the fractional attainment of equilibrium as a function of time. Although bilirubin was reasonably stable during the binding studies, attempts to achieve equilibrium would probably have resulted in the eventual disappearance of bilirubin from the solution as a consequence of binding to the resin and the decomposition of bilirubin in solution. For the latter reason, and since long equilibrium times would be involved where the binding rates were very low (27), and also because of the complexity of the system, an empirical rate expression was sought for the binding process. A second-order rate expression was found to be satisfactory for the present studies (28, 29) as can be seen in Fig. 1. The mathematical expression which suitably characterizes the rate of binding to resins for fast processes, and initial stages of slow processes is:

$$\ln \frac{b\,(a - x)}{a\,(b - x)} = (a - b)\,kt$$

where

a = the initial number of meq. of bilirubin in solution,

b = the initial number of meq. of ion-exchange resin in the reaction flask based on the weight of wet resin used, the moisture content of the resin, and the experimentally determined weight capacity,

$a - x$ = the number of meq. of bilirubin remaining in solution at time t,

$b - x$ = the number of meq. of ion-exchange resin remaining in the chloride form at time t,

k = second-order rate constant, 1./(meq. hr.),

t = time in hr.

It was assumed that bilirubin was bound by ion exchange and that it existed as a divalent anion under the conditions employed with an equivalent weight of 292.

The second-order rate constants were calculated from the initial slope of the lines (0 to approx. 12 hr.) obtained by plotting log $[b(a - x)]/[a(b - x)]$ against t as shown in Fig. 1. In order to obtain k in the proper units it is necessary to multiply the slope by $[2.303/(a - b)]$ [190/1,000] to change from decadic to natural logarithms, and to account for the experimental volume of 190 ml. The averages of the rate constants determined in duplicate for each resin are given in Table I.

While the binding of bilirubin obeys a second-order rate law as mentioned previously, plots over longer periods of time for slow processes, *i.e.*, binding of bilirubin to resins with a large mesh size and a high degree of crosslinking, show that the experimental points deviate from linearity with decreasing slopes as time increases. This can be explained if it is assumed that the capacity of the resin for bilirubin is less than that for the chloride ion. The reduction in capacity could be due to insufficient space within the resin structure for the accommodation of the bilirubin ion in a concentration equivalent to the total exchange capacity of the resin, even if each exchange site is accessible to the organic ion at low concentrations of bilirubin ion in the resin phase. Another explanation which has been suggested is that some regions of the resin phase are completely inaccessible to the bilirubin ion even at low concentration of the organic ion in the resin (30).

If the uptake of bilirubin by an ion-exchange resin were to be considered strictly as a chemical reaction, two ion exchangers differing only in particle size should bind the pigment at the same rate. It can be seen from Table I that this is not the case for the ion-exchange[2] resins of different mesh sizes. Particle size has a marked effect on the rate constant indicating that chemical reaction at the exchange sites is not the rate-controlling step. If the rate is controlled by diffusion into the particle it should vary as $1/r^2$. If, however, film diffusion is the controlling factor the rate should vary as $1/r$ (31). It can be seen from Table I that the rate constant k generally increases with decreasing bead radius for resins with the same crosslinking. A consideration of the 1-X4 resins indicates that the rate is primarily particle-diffusion controlled because the product kr^2 has less variation than the product kr.

The other variable studied in this research was the influence of the degree of crosslinking of the resin on the rate of binding. The crosslinking of the resin determines the swelling and the moisture content of the resin beads. Table I shows that the rate constant k increases with decreasing crosslinking for resins of the same mesh size. Resins with low crosslinking swell more in an aqueous medium and thus contain a larger proportion of water. As a result more exchange sites are available to the large organic ion. Furthermore, the fractional pore volume of the ion exchanger is increased; hence retardation of the framework will be less. Resins with a high degree of crosslinking were found to have low moisture contents and low rates of binding (Table I). A comparison of resins of the same particle size and crosslinking shows that the moisture contents of Type 1 resins are higher than those determined for Type 2 resins, however only the resins with 4% divinylbenzene content have rates of binding which are in agreement with the previous

[2] Dowex 1-X4.

statement. The effect of crosslinking on the rate of binding can be interpreted by considering that the fractional moisture content equals the fractional pore volume of the ion exchanger. The effect of the resin matrix is to retard diffusion and thus decrease the rate of binding. The reasons given by Helfferich (32) for this phenomenon are: (*a*) part of the cross-section in the ion exchanger is occupied by the polymer chains and is not available for diffusion, (*b*) the diffusion paths in the resin are more tortuous and therefore longer, (*c*) large ions or molecules in the ion exchanger may be impeded in their mobility by the framework, (*d*) interaction with fixed ionic groups may retard counterion diffusion. A quasi-homogeneous model has been described which relates diffusion coefficients in ion exchangers to those in solutions, and the diffusion coefficients have been found to depend upon the fractional pore volume of the medium (33).

In order to obtain a correlation between the second-order rate constants and both the radius and the fractional pore volume (ϵ) of the resin beads, several relationships were considered. It was found that a linear relation could be obtained by plotting kr^2 against $[\epsilon/(2 - \epsilon)]^2$ as shown in Fig. 2.

This indicates that the binding of bilirubin by anion-exchange resins is controlled by particle diffusion in this experiment, since the rate is dependent on the square of the radius and the fractional pore volume of the ion-exchange resin. Because of the dependence of the rate on the term $[\epsilon/(2 - \epsilon)]^2$ rather than $\epsilon/2$, it can be assumed that the decrease in rate is caused not only by a reduction in cross-sectional area but also by diffusion paths which are more tortuous (34).

It is not possible at this time to give a satisfactory explanation for the behavior of the 1-X4 (200–400) resin. The rate-limiting step for binding may no longer be controlled mainly by particle diffusion, as

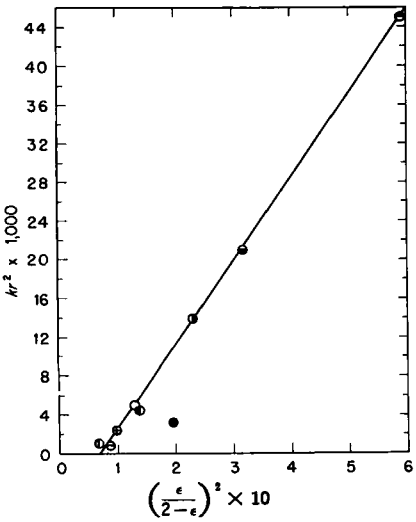

Fig. 2—Plot illustrating the relationship between the second-order rate constant, the radius, and the fractional pore volume. Key: ◓, *1-X1 (50–100 mesh);* ◒, *1-X2 (50–100 mesh);* ○, *1-X4 (20–50 mesh);* ◐, *1-X4 (50–100 mesh);* ◑, *1-X4 (100–200 mesh);* ●, *1-X4 (200–400 mesh);* ⊕, *2-X4 (20–50 mesh);* ⊖, *1-X8 (50–100 mesh);* ⊙, *2-X8 (50–100 mesh).*

the importance of film diffusion could increase as the particle size decreases (35).

Since oral administration of an ion exchanger would allow only a limited time of contact of the resin with intestinal contents, the rate of binding of bilirubin by the exchanger should be high if the resin is to be an effective therapeutic agent. On the assumption that there would be some correlation between the *in vitro* binding rates determined by this research and the rates that might occur under physiological conditions, the most effective anion-exchange resin for reducing high serum levels of bilirubin would be a Type 1 resin of low crosslinking and small bead diameter. It is to be expected that *in vivo* conditions, such as a low degree of agitation and the presence of competing ions, will alter pigment-binding rates. Nevertheless, it is quite conceivable that, as therapeutic agents, these resins would exhibit the same relative order of binding rates as was determined in this study.

REFERENCES

(1) Lester, R., Hammaker, L., and Schmid, R., *Lancet*, **2**, 1257(1962).
(2) Visintine, R. E., Michaels, G. D., Fukayama, G., Conklin, J., and Kinsell, L. W., *ibid.*, **2**, 341(1961).
(3) Lottsfeldt, F. I., Krivit, W., Aust, J. B., and Carey, J. B., Jr., *New Engl. J. Med.*, **469**, 186(1963).
(4) Van Itallie, T. B., Hashim, S. A., Crampton, R. S., and Tennent, D. M., *ibid.*, **265**, 469(1961).
(5) Tennent, D. M., Siegel, H., Zanetti, M. E., Kuron, G. W., Ott, W. H., and Wolf, F. J., *J. Lipid Res.*, **1**, 469 (1960).
(6) Schmid, R., Diamond, I., Hammaker, L., and Gundersen, C. B., *Nature*, **206**, 1041(1965).
(7) Sakamoto, T., Yamamoto, S., Yahata, K., and Kondo, T., *Igaku Kenkyu*, **27**, 373(1957).
(8) Schmid, R., in "The Biliary System," Taylor, W., Ed., Blackwell Scientific Publications, Oxford, England, 1965, p. 229.
(9) Beccari, E., *Boll. Soc. Ital. Biol. Sper.*, **4**, 1273(1929); through *Chem. Abstr.*, **24**, 3255(1930).
(10) Beccari, E., *Arch. Fisiol.*, **28**, 452(1930).
(11) Boutaric, A., and Roy, M., *Bull. Soc. Chim. Biol.*, **25** 30(1943); through *Chem. Abstr.*, **38**, 1688²(1944).
(12) Barac, G., and Roseman, R., *J. Wash. Acad. Sci.*, **36**, 296(1946).
(13) Najjar, V. A., and Childs, B., *J. Biol. Chem.*, **204**, 359(1953).
(14) Fog, J., and Bugge-Asperheim, B., *Nature*, **203**, 756(1964).
(15) deEwenson, I. W., Gianturco, F. A., and Gramaccioni, P., *Experientia*, **22**, 14(1966).
(16) Clarke, J. T., *Clin. Chem.*, **11**, 681(1965).
(17) Schellong, G., and Wende, U., *Klin. Wochschr.*, **38**, 703(1960); through *Chem. Abstr.*, **54**, 24984*b*(1960).
(18) Meites, S., and Traubert, J. W., *Clin. Chem.*, **11**, 691(1965).
(19) Barac, G., *Bull. Soc. Chim. Biol.*, **21**, 1163(1939); through *Chem. Abstr.*, **34**, 2006⁵(1940).
(20) Lambrechts, A., Barac, G., *ibid.*, **21**, 1171(1939); through *Chem. Abstr.*, **34**, 2006⁶(1940).
(21) Barac, G., and Roseman, R., *Compt. Rend. Soc. Biol.*, **140**, 581(1946); through *Chem. Abstr.*, **41**, 2094*b*(1947).
(22) Henry, R. J., Jacobs, S. L., and Chiamori, N., *Clin. Chem.*, **6**, 529(1960).
(23) Burnstine, R. C., and Schmid, R., *Proc. Soc. Exptl. Biol. Med.*, **109**, 356(1962).
(24) Sawchuk, R. J., Anderson, J. M., and Nairn, J. G., *J. Pharm. Sci.*, **55**, 1463(1966).
(25) College of American Pathologists, Standards Committee, *Am. J. Clin. Pathol.*, **39**, 90(1963).
(26) Helfferich, F., "Ion Exchange," McGraw-Hill, New York, N. Y., 1962, p. 251.
(27) Gregor, H. P., Bregman, J. I., Gutoff, F., Broadley, R. D., Baldwin, D. E., and Overberger, C. G., *J. Colloid. Sci.*, **6**, 20(1951).
(28) Nachod, F. C., and Wood, W., *J. Am. Chem. Soc.*, **66**, 1380(1944).
(29) Helfferich, F., *Reference 26*, p. 286.
(30) Dmitryenko, L. V., and Hale, D. K., *J. Chem. Soc.*, **1965**, 5570.
(31) Helfferich, F., *Reference 26*, p. 285.
(32) Helfferich, F., in "Ion Exchange," Vol. 1, Marinsky, J. A., Ed., Marcel Dekker, New York, N. Y., 1966, p. 69.
(33) Helfferich, F., *Ibid.*, p. 72.
(34) Helfferich, F., *Reference 26*, p. 302.
(35) *Ibid.*, p. 255.
(36) "Dowex:: Ion Exchange," The Dow Chemical Company, Midland, Mich., 1964.

Keyphrases

Ion-exchange resin—binding
Bilirubin binding—ion-exchange resins
Kinetics—bilirubin-resin binding
Colorimetric analysis—spectrophotometer

Modified Confinement Motor Activity Test For Use in Mice

By GERALD MILNER

The modification of a confinement motor activity (CMA) unit, for use with mice, is described. Its use in quantitating the effect of drugs on the motor activity in mice is illustrated by experiments with amitriptyline and alcohol. Amitriptyline (10 mg./kg.), given orally, was found to significantly increase CMA when compared with control groups. Alcohol (25 ml./kg., 10% solution) depressed CMA in mice, but not to a statistically significant extent. When the amitriptyline and alcohol were given together, a significant depression of CMA was recorded. A positive joint action is suggested—amitriptyline, when taken by man, may add to the effects of alcohol.

MEASUREMENTS OF alterations in locomotor activity have proved valuable in testing for drug effects on animal behavior. In 1964 Tedeschi *et al.* described a photoelectric cell counting chamber (1) for measuring confinement motor activity (CMA) in rats. This consisted of a chamber so small as to prevent the rat moving from place to place, but high enough to permit the typical "up-and-down" exploratory behavior of the confined rodent. The rat's movements were counted by means of two photoelectric cells. The advantages of the CMA chamber over conventional locomotor activity units are that it is more compact, cheaper, and more sensitive—particularly when testing stimulant drugs such as caffeine, tranylcypromine, and amphetamine. Photoelectric cell activity chambers have been shown to be generally more satisfactory than mechanical tests, such as those involving rocking floors (2).

Mice are probably the most widely used and useful laboratory animals; they are small, easily maintained and bred, cheap, and can be used in large numbers for individual experiments. With many drugs, particularly alcohol, prediction of behavioral effects in man from their effects in mice have proved valid (3). The present paper describes the development of a confinement motor activity chamber for mice. Its use is illustrated by experiments involving amitriptyline and alcohol.

Amitriptyline is an iminodibenzyl drug (with a chemical formula closely related to chlorpromazine) and in doses of 30 and 50 mg./kg. body weight has been shown to significantly increase the length of loss of righting reflexes in mice given alcohol (4, 5). With alcohol levels of 25 ml./kg. of a 25% solution, the average length of loss of righting reflexes in an adult mouse is 1.2 hr., whereas a dose of 25 ml./kg. of 20% alcohol just fails to cause coma. The significant increase in the length of alcoholic coma brought about by amitriptyline might be due only to the sedation caused by each drug. A positive joint action of alcohol and amitriptyline is indicated in the experiments described in this paper, where low doses of the drugs were used.

EXPERIMENTAL

Development of a CMA Unit for Mice—Preliminary experiments enabled a rough estimate of the best size of chamber to be made. Tests were then

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This work forms part of a thesis submitted for the Doctor of Medicine degree, University of Western Australia.