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Protein binding and the excretion of some azo dyes in rat bile

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The kinetics of biliary excretion of four dyes, amaranth, geranine, lissamine and dechlorolissamine have been examined in the rat. The protein binding of each dye to blood and liver *in vitro* was examined, and a correlation found between excretion rate and the relative degree of binding of dye to the liver compared with the blood.

THE ability of a compound to be excreted in the bile appears to be related to molecular size and metabolic pathway (Williams, Millburn & Smith, 1965). The transport of material from the blood to the bile often involves active transport processes, for example, with anions (Sperber, 1959) and some cations (Schanker & Solomon, 1963).

Ryan & Wright (1961, 1962) reported the almost quantitative biliary excretion of some water-soluble azo dyes. In contrast to the compounds studied by Williams & others (1965), these dyes were excreted unchanged. There appeared to be no correlation between the amount of dye excreted within 6 hr of intravenous injection and chemical structure or molecular weight.

Few studies on the biliary excretion of drugs have involved the determination of the kinetics of the process. These azo dyes, rapidly excreted and not metabolized, would appear to be ideal compounds for use in the study of biliary excretion kinetics. Four dyes were chosen and an attempt made to determine their biliary excretion kinetics and to relate this to their tendency to bind to protein both in the liver and in the blood.

Experimental

MATERIALS

Three of the dyes, amaranth (Colour Index No. 16185), geranine 2GS (Colour Index No. 18050) and lissamine fast yellow 2 G (Colour Index No. 18965) were commercial samples, recrystallised from ethanol: water. Dechlorolissamine [the di-sodium salt of 1-(*p*-sulphophenyl-)3-methyl-4-(*p*-sulphophenylazo-)5-pyrazolone] was kindly supplied by Dr. A. J. Ryan. All dyes were chromatographically and analytically pure.

METHOD

Collection of bile. Albino male rats (250-350 g) were anaesthetised with urethane (125 mg/100 g). A longitudinal incision was made just below the diaphragm, and the bile duct isolated. After ligation of the duct at the duodenal end, a cannula consisting of a 23 gauge hypodermic needle shaft attached to a length of polyethylene tube was introduced into the bile duct, and secured with cotton thread. The dose of dye was injected, in aqueous solution, into a femoral vein and bile was collected

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at 5–15 min intervals for 2–2½ hr. The body temperature of the rats was maintained by exposure to a radiator.

Analysis of samples. The dye content of the bile samples was determined by diluting the samples with 0·01M hydrochloric acid to suitable volume, and reading the optical density in a Bausch and Lomb Spectronic 20 spectrophotometer at the wavelength of maximum absorption. Where bile pigments interfered with the optical density readings, these were precipitated by adding equal volumes of 40% zinc sulphate solution and 11·2% potassium hydroxide solution, eluting the dye from the precipitate by washing with hot water before adjusting to a suitable volume with dilute acid.

PROTEIN BINDING

The degree of protein binding in both whole rat blood and rat liver homogenates was determined *in vitro* for the four azo dyes. 4·0 ml of dye (2×10^{-4}M) in Krebs-Ringer-phosphate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1957), and 4·0 ml of the blood or liver homogenate, diluted to 14 mg/ml protein (equivalent to bovine serum albumin), as a standard with Krebs-Ringer-phosphate buffer pH 7·4, were placed in a centrifuge tube and shaken at 37° for 10 min. 1·0 ml of 40% zinc sulphate solution and 1·0 ml 11·2% potassium hydroxide solution were added to precipitate the protein and protein-bound dye. The mixtures were centrifuged and the supernatant diluted and read in a spectrophotometer, to determine free dye. Rat liver homogenates were prepared in an all-glass Dounce homogeniser with a loose-fitting, unground plunger, and both blood samples and liver homogenates were standardised for protein concentrations by the biuret method of Cleland & Slater (1953).

The method of protein precipitation outlined above was used because it involves only brief exposure of the preparation to extremes of pH. Thus it gives a more physiological measure of protein binding than precipitation with trichloroacetic acid, where acidic conditions may abstract some bound dye from the protein. The purpose of the binding experiments used here was to determine the relative binding capacities of liver and blood and not the absolute capacity in each case. For this reason a fixed concentration of dye was reacted with blood and liver preparations standardised to the same amount of protein.

Results and discussion

The dyes appear in the biliary cannula within 3–5 min of injection. Fig. 1 shows the kinetic analysis of results obtained with the dyes after a dose of 20μmoles had been administered in each case. Two types of semilogarithmic plots can be used with cumulative excretion results of this type (Wagner, 1963). The more common plot of log amount not excreted [log (a-x)] against time, requires that the total amount excreted (a) be known. During the time of biliary collection it was difficult to make an accurate estimate of (a). The method used was a plot of log excretion

rate against the mid-point of the collection interval. This plot is independent of the total amount excreted and the first order rate constant can be calculated from the slope of the curve.

Fig. 1 indicates that the excretion process may be described by two first order processes, an initial rapid excretion of most of the dye followed by a slower residual excretion. This biphasic excretion may be related to changes in bile flow rate or to storage of some of the dye in a depot from which slow release occurs after the unbound dye has been excreted. Wiseman, Schreiber & Pinson (1964) have reported a similar biphasic curve in their study of blood levels of benzquinamide and explain the slow secondary release of drug as due to release of unchanged drug or metabolites from tissue depots. Richard, Tyndall & Young (1959) also obtained a similar biphasic curve in studies on rate of plasma disappearance of sulphobromophthalein sodium but their measurements of biliary excretion cover an insufficient time to allow any indication of biphasic excretion to become apparent. Hence biphasic liver uptake may or may not be related to biphasic excretion and more work is required to clarify this point.

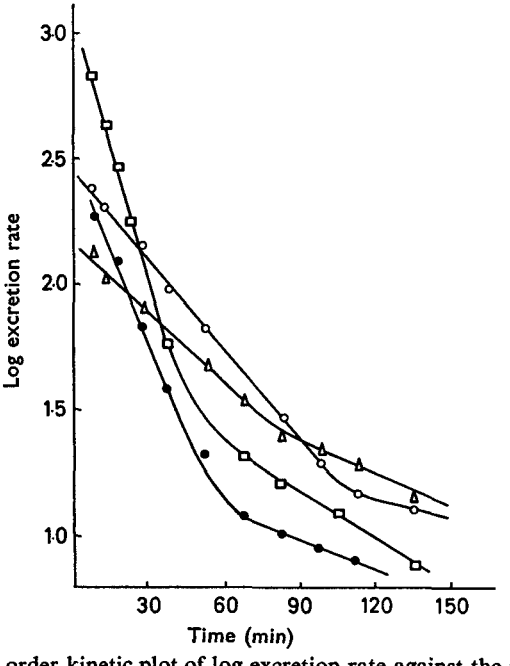

Fig. 1. First-order kinetic plot of log excretion rate against the mid-point of the collection intervals for the four azo dyes: □ Amaranth, ● Geranine, ○ Lissamine, △ Dechlorolissamine

Inspection of Table 1 shows that there is no simple relationship between the excretion rate and molecular weight of the dye anion nor do the rate constants show any correlation with the final total excretion.

Brauer & Pessotti (1949) showed that sulphobromophthalein sodium was bound to liver slices *in vitro* and also addition of bovine serum albumin reduced the degree of binding. This result indicates that the dye can be bound by liver and that plasma protein can reduce the degree of liver binding. Andrews & del Rio Lozano (1961) confirmed these results in perfused preparations. These studies suggest that protein binding in the liver could be important in the hepatic uptake, if not excretion, of compounds. To test this possibility, the ability of liver and blood preparations to bind dyes was examined. The technique used measures the amount of unbound dye left in a supernatant after precipitation of the protein contained in the system. With both liver and blood the dyes used were bound to protein to a large extent (Table 1). A relationship is apparent between excretion rate and liver: blood binding ratio (Table 1). Amaranth, the dye most strongly bound by the liver in preference to the blood, is the one most rapidly excreted. If the relative liver: blood binding ratios and excretion rates of the four dyes are compared by setting the values for dechlorolissamine at unity, then the correlation of preferential protein binding and excretion rate is obvious. For amaranth the respective figures are 2·19, 2·50; for geranine, 1·79, 1·99; for lissamine, 1·25, 1·1.

TABLE 1. KINETIC DATA FOR THE INITIAL, RAPID EXCRETION PHASE FOR ALL DYES AT A DOSE LEVEL OF 20 μMOLES AND RESULTS FOR PROTEIN BINDING IN RAT LIVER HOMOGENATES AND WHOLE RAT BLOOD

Dye	Amaranth	Geranine	Lissamine	Dechloro-lissamine
Mol. wt. of dye anion	535	463	505	436
Mean rate constant k_1 (min^{-1}) ..	0·0634	0·0507	0·028*	0·0254
Excretion half-life, $t_{\frac{1}{2}}$ (min)	10·9	13·7	24·7*	30·0
Mean % biliary excretion in 6 hr† ..	53	46	96	80
Protein bound in liver (%)‡	91·4 ± 1·1	57·2 ± 3·3	50·3 ± 3·7	43·6 ± 3·0
Protein bound in blood (%)‡	81·4 ± 4·1	62·4 ± 4·7	78·0 ± 7·3	85·0 ± 1·4
Liver: blood binding ratio	1·123	0·917	0·645	0·513

* The excretion rate constant of lissamine varies with the dose given while for the other dyes the rate constant maintains the same value until high saturation doses are reached (100 μmoles) (Priestly, 1965).
† These values are quoted from Ryan & Wright (1961, 1962).
‡ Average of six determinations each.

The method of determining protein binding is somewhat artificial and measures an equilibrium static situation compared with the kinetic situation of dye excretion. Nevertheless, it can be concluded that with the dyes studied, their rate of biliary excretion appears to be a function of their relative degree of binding to the liver proteins as against blood proteins. It would seem that dye protein binding in the liver is an integral part of the excretion process and not a storage phase delaying excretion, as appears to be the case with sulphobromophthalein sodium (Andrews & del Rio Lozano, 1961).

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