Biochemical Pharmacology, Vol. 22, pp. 2743–2751. Pergamon Press, 1973. Printed in Great Britain.

STUDIES ON THE BILIARY EXCRETION MECHANISM OF DRUGS—I

BILIARY EXCRETION OF AZO DYES IN THE RAT

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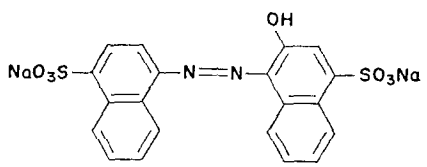
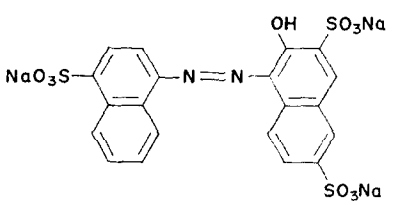
(*Received* 10 *August* 1972; *accepted* 27 *April* 1973)

Abstract—The mechanism of biliary excretion of the azo dyes, Azorubin S (AS), Amaranth (AM) and New Coccine (NC), in rats was investigated. It was observed that these azo dyes have an apparent transport maximum (Tm) for their biliary excretion. Further, the biliary excretion of these dyes was markedly depressed by phenolphthalein glucuronide (PPG) and probenecid, which are considered to be actively excreted into bile. The results, therefore, suggest that the biliary excretion of these dyes in rats involves an active transport process.

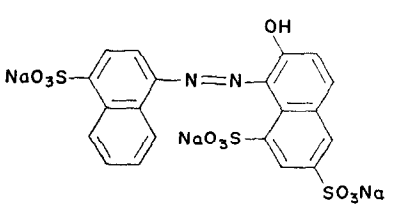
IN RECENT years, numerous investigations have been performed on the biliary excretion of azo dyes. Azorubin S (AS), Amaranth (AM) and New Coccine (NC), containing sulfonic acid groups (Fig. 1), have been shown to be excreted in large



Azorubin S (AS); mol. wt, 502·45



Amaranth (AM); mol. wt, 604·5



New coccine (NC); mol. wt, 604·5

FIG. 1. Chemical structure of azo dyes.

quantities in the bile of rats in the unchanged form. However, little is known about the mechanism by which they are excreted. The present report describes the biliary excretion rates of the dyes and the effects of other organic anions on excretion of the dyes.

METHODS AND MATERIALS

Procedure in animals. Male Wistar strain rats, weighing 280–320 g, were anesthetized with sodium pentobarbital intraperitoneally (40 mg/kg of body wt). Through a midline abdominal incision, the renal pedicles were ligated to prevent renal excretion, and the bile duct was then cannulated with polyethylene tubing. A thermistor probe was placed on the surface of liver, and body temperature was maintained throughout the experiments at 38° with a heating lamp. After the incision had been closed, the drugs were administered intravenously via the femoral vein. In some experiments, either phenolphthalein glucuronide (PPG) or probenecid was infused together with these dyes. Bile was then collected for 2-min periods for 30 min, and then for 10-min periods for an additional 60 min.

Thin-layer chromatography. Chromatography was carried out on bile which had been collected for 1 hr from a rat with ligated renal pedicles after intravenous adminstration of each dye (50 μmoles). Chromatograms were developed on Silica gel plates (Merck, Kieselgel HF_{254}) with the following two solvent systems: (A) *n*-propanol–ethyl acetate–water (6:1:3, v/v); (B) *n*-butanol–ethanol–28% ammonium hydroxide solution–water (4:4:1:2, v/v). All chromatograms were run a distance of 13 cm at room temperature. The following two tests were used: (1) ultraviolet light and (2) the Ehrlich reagent test. In the first, chromatograms were examined under ultraviolet light (254 nm). Some compounds quenched the background fluorescence of the plate and appeared as dark spots. The Ehrlich reagent test was carried out to detect primary amines. A solution was made of 1 g *p*-dimethylaminobenzaldehyde in a mixture of 25 ml hydrochloric acid (36%, w/v) and 75 ml methanol.

The R_f-values of three azo dyes in rat bile are shown in Table 1. All chromatograms of the bile of rats treated with AS or NC revealed only a single spot, and the R_f-value in each system was identical to that of authentic dye. On the other hand, in the case of AM, a pink spot which was considered as a metabolite was observed above a spot

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF AZO DYES*

Compound	R_f-values		Fluorescence in u.v. light (254 nm)	Colour
	Solvent A	Solvent B		
Azorubin S (AS)	0·88	0·63	Q†	Red
Amaranth (AM)	0·78	0·55	Q	Red–purple
Biliary metabolite of AM‡	0·90	0·66	Q	Pink
New Coccine (NC)	0·84	0·52	Q	Orange

* Thin-layer chromatography (TLC) was carried out on bile samples which had been collected for 1 hr from a rat with ligated renal pedicles after administration of dye (50 μmoles).

† Q = quenches background fluorescence of TLC plate.

‡ The ratio of the biliary metabolite of AM to unchanged AM was less than 1 per cent as determined by absorbance at 522 nm.

corresponding to the unchanged dye. Each spot corresponding to unchanged AM and the unknown metabolite of AM was scraped from the plate, suspended in water, and centrifuged at 2000 rev/min for 5 min. The absorbance of the supernatant at 522 nm was compared.

Determination of dyes. Bile and plasma samples were diluted with water and the absorbance of the solution was measured at 522 nm for AM, at 515 nm for AS, and at 507 nm for NC, against appropriate blanks. Under the conditions of this assay, the recovery of dyes was quantitative, and the bile and the plasma blanks were negligible.

Determination of PPG. Determination of PPG was carried out with a modified method of Millburn *et al.*[1] One ml of sample solution containing 5–80 μg/ml was heated with an equal volume of 8 N HCl in a boiling water bath for 1 hr. The solution was neutralized with 4 N NaOH, and then slightly alkalinized with 0·5 N NaOH. The resulting solution was made up to 10 ml with glycine buffer (pH 10·4) prepared just before use, and the color measured at 550 nm.

The samples containing azo dyes were treated with 8 N HCl containing 0·05% stannous chloride. By this treatment, these dyes were reduced and decolorized completely. The treatment did not influence the measurement of PPG, and the recovery of PPG (5–80 μg/ml) added to bile was about 100 per cent.

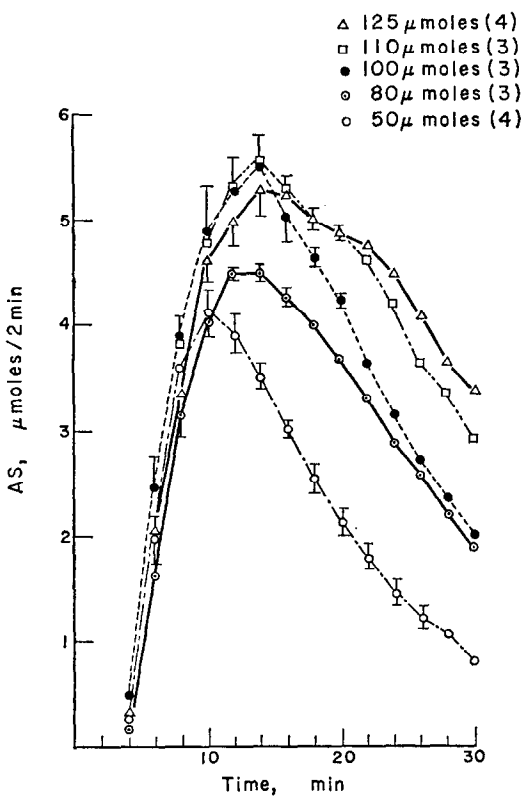


FIG. 2. Biliary excretion of various doses of AS after i.v. administration in rats with ligated renal pedicles. Bile was collected for 2-min periods for 30 min. Number of experiments is shown in parentheses. Results are given as the mean ± S.E.

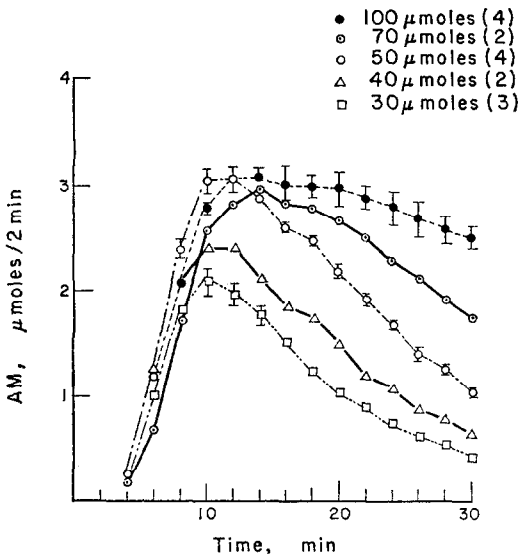


FIG. 3. Biliary excretion of various doses of AM after i.v. administration in rats with ligated renal pedicles. Bile was collected for 2-min periods for 30 min. Number of experiments is shown in parentheses. Results are given as the mean ± S.E.

Drugs. All azo dyes were obtained from Tokyo Chemical Industries, Company, Ltd., and recrystallized from ethanol–water mixture. PPG was obtained from Sigma Chemical Company. Probenecid, m.p. 194–196°, was used.

Instrument. A model 124 Hitachi spectrophotometer was used.

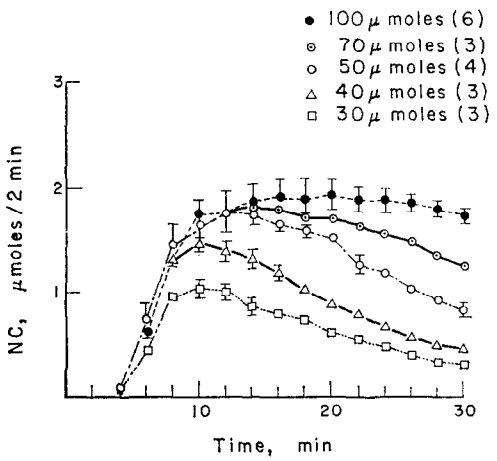


FIG. 4. Biliary excretion of various doses of NC after i.v. administration in rats with ligated renal pedicles. Bile was collected for 2-min periods for 30 min. Number of experiments is shown in parentheses. Results are given as the mean ± S.E.

RESULTS

Saturation of the biliary excretion process. Evidence that AS, AM and NC are transported into bile by a saturable process was obtained from a study of the biliary excretion of the dyes at different dose levels.

Biliary excretion of AS. Figure 2 shows the biliary excretion of AS at various doses ranging from 50 to 125 μmoles. Bile was collected for 2-min periods for 30 min. The maximal excretion rates observed at doses of 100, 110 and 125 μmoles were essentially identical.

Biliary excretion of AM. Figure 3 shows the biliary excretion rates (μmoles/2 min) of AM after intravenous administration at various doses ranging from 30 to 100 μmoles. The maximal excretion rates observed at doses of 50, 70 and 100 μmoles were essentially identical.

Biliary excretion of NC. Figure 4 shows the excretion rates (μmoles/2 min) of NC at various doses ranging from 30 to 100 μmoles. The maximal excretion rates observed at doses of 50, 70 and 100 μmoles were essentially identical.

Bile-to-plasma concentration ratios of dyes. The comparison between the concentrations in bile and plasma was carried out at the time when the excretion rates of these dyes were maximal. Bile was collected for 2 min from 10 to 12 min after injection. Blood was collected from the carotid in a heparinized beaker at the end of the bile collection. In Table 2, the concentrations of AS, AM and NC in plasma and bile are shown. The results show that the concentrations of these dyes in bile greatly exceed those in plasma at 12 min, especially at the lower doses.

TABLE 2. CONCENTRATION OF DYES IN BILE AND PLASMA*

Dye	Dose (μmoles)	Body wt of animal (g)	Plasma (μmoles/ml)	Bile (μmoles/ml)	Bile-to-plasma concn ratio
AS	50	315	0·71	59·0	83·0
AS	100	310	1·39	67·3	48·4
AM	50	330	0·21	32·4	154·3
AM	100	320	0·67	36·0	53·7
NC	50	310	0·47	24·1	51·3
NC	100	340	0·99	26·1	26·4

* Rats with ligated renal pedicles received azo dyes, 50 and 100 μmoles i.v. Each value represents the results in one animal. The bile was collected for 2 min from 10 to 12 min after injection. Blood was collected from the carotid in a heparinized beaker at the end of the bile collection.

Depression of biliary excretion of AS by PPG and probenecid. As shown in Fig. 5(a) and (b), the excretion of AS at a dose of 50 μmoles was depressed by PPG and probenecid at the same dose as that of AS. The maximal excretion rate (4·14 ± 1·04 μmoles/2 min) of AS was depressed 69·1 per cent ($P < 0·05$) by PPG, and 24·1 per cent ($P < 0·05$) by probenecid. The total amount of excretion (39·5 ± 1·04 μmoles) in 90 min was depressed 20·2 per cent ($P < 0·05$) by PPG. On the other hand, the amount of excretion in 90 min in probenecid-treated animals was 37·1 ± 1·33 μmoles, not significantly different ($P > 0·05$) from that of controls.

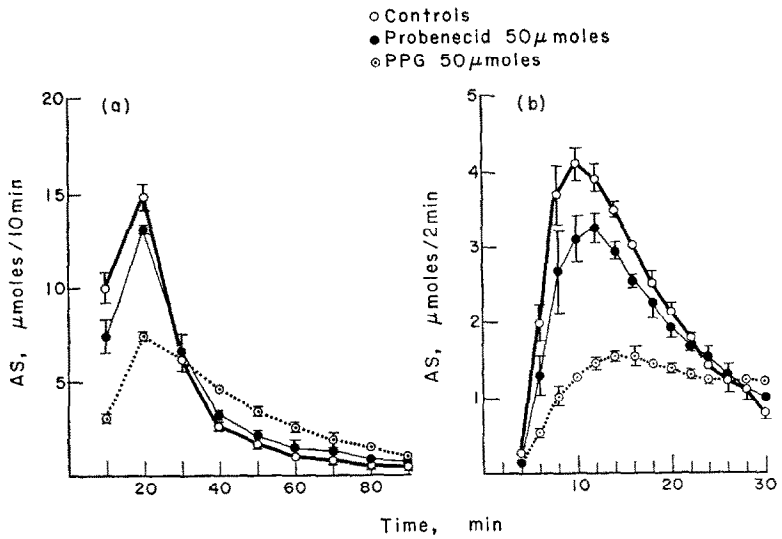


FIG. 5. Effects of PPG and probenecid on biliary excretion of AS. The excretion rate of AS is plotted against time when administered (AS, 50 μmoles) together with either PPG (50 μmoles) or probenecid (50 μmoles). Bile was collected for 2-min periods for 30 min, and then for 10-min periods for an additional 60 min. Each result is given as the mean ± S.E. for four animals. (a) Shows the excretion rates for 10-min periods (μmoles/10 min); (b) shows the excretion rates for 2-min periods (μmoles/2 min).

Depression of biliary excretion of AM by PPG and probenecid. In Fig. 6(a) and (b), the effects of PPG and probenecid on the excretion of AM at a dose of 50 μmoles are shown. As seen also for AS, the inhibitory effect by PPG was more marked than that by probenecid. The maximal excretion rate (3·12 ± 0·03 μmoles/2 min) of AM was

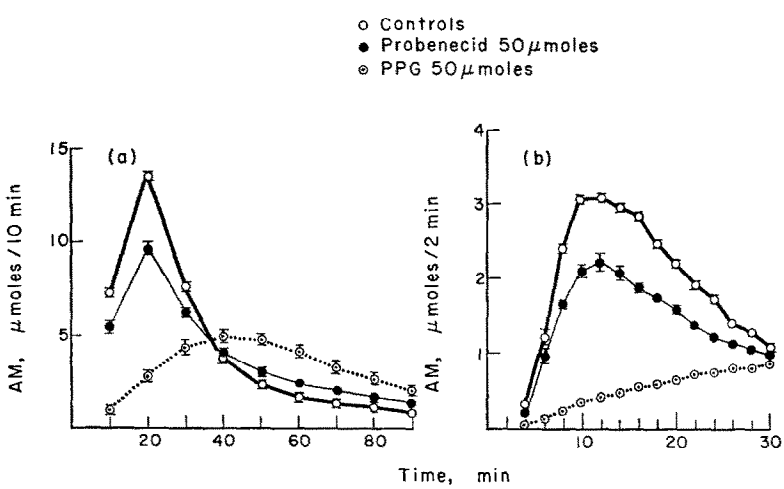


FIG. 6. Effects of PPG and probenecid on biliary excretion of AM. Bile was collected for 2-min periods for 30 min, and then for 10-min periods for an additional 60 min. Each result is given as the mean ± S.E. for four animals. (a) Shows the excretion rates for 10-min periods (μmoles/10 min); (b) shows the excretion rates for 2-min periods (μmoles/2 min).

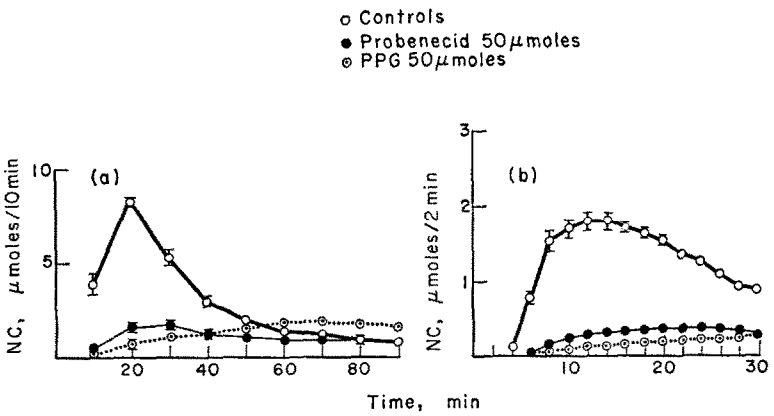


FIG. 7. Effects of PPG and probenecid on biliary excretion of NC. Bile was collected for 2-min periods for 30 min, and then for 10-min periods for an additional 60 min. Each result is given as the mean ± S.E. for four animals. (a) Shows the excretion rates for 10-min periods (μmoles/10 min); (b) shows the excretion rates for 2-min periods (μmoles/2 min).

depressed 87·3 per cent ($P < 0.05$) by PPG, and 28·0 per cent ($P < 0.05$) by probenecid. The total amount of excretion (37·1 ± 0·42 μmoles) in 90 min was depressed 28·3 per cent ($P < 0.05$) by PPG, and 12·2 per cent ($P < 0.05$) by probenecid.

Depression of biliary excretion of NC by PPG and probenecid. In Fig. 7 (a) and (b), the effects of PPG and probenecid on the excretion of NC at a dose of 50 μmoles are shown. The maximal excretion rate (1·86 ± 0·06 μmoles/2 min) of NC was depressed 93·9 per cent ($P < 0.05$) by PPG, and 86·1 per cent ($P < 0.05$) by probenecid. The total amount of excretion (26·5 ± 0·73 μmoles) in 90 min was depressed 63·8 per cent ($P < 0.05$) by PPG, and 53·4 per cent ($P < 0.05$) by probenecid. It was observed that the biliary excretion of NC was most markedly depressed by PPG and probenecid among these dyes.

Failure of dyes to influence biliary excretion of PPG. It was described above that the biliary excretion of dyes was depressed by PPG and probenecid. In contrast, the biliary excretion of PPG at a dose of 50 μmoles was not influenced by these dyes, as shown in Fig. 8 (a) and (b). The maximal excretion rate of PPG (μmoles/2 min) in

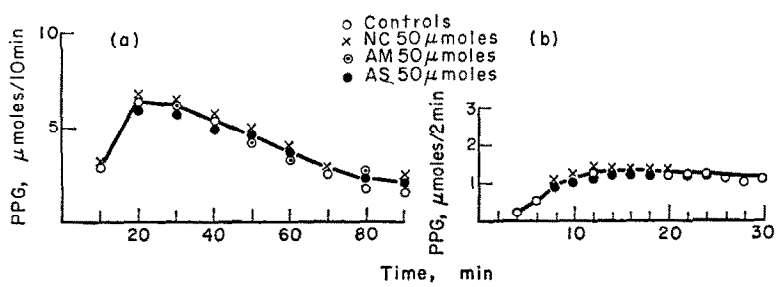


FIG. 8. Effects of AS, AM and NC on biliary excretion of PPG. Each result is given as the mean ± S.E. for four animals. (a) Shows the excretion rates for 10-min periods (μmoles/10 min); (b) shows the excretion rates for 2-min periods (μmoles/2 min).

dye-treated animals at this dose (50 μmoles) was not significantly different from that of controls (1·33 ± 0·08 μmoles/2 min); 1·18 ± 0·08 μmoles/2 min in AS-treated animals ($P > 0·9$), 1·28 ± 0·07 μmoles/2 min in AM-treated animals ($P > 0·4$), and 1·39 ± 0·03 μmoles/2 min in NC-treated animals ($P > 0·3$). These results suggest that these dyes had little influence on the transport of PPG.

DISCUSSION

The present studies indicate that AS, AM and NC are actively secreted into bile. This conclusion is based on the following evidence: these azo dyes are transferred from plasma to bile against a large concentration gradient; the transfer process is saturable; and excretion of these dyes is inhibited by phenolphthalein glucuronide (PPG) and probenecid.

It has been reported by Radomski and Mellinger[2] that, after oral administration of AM, 1-amino-4-naphthalene disulfonic acid is excreted into the urine and bile after fission of the azo linkage.

In the present studies, metabolites of azo dyes excreted in the bile were investigated using thin-layer chromatography with two solvent systems (Table 1). In the case of AM, a pink spot, which was considered as a metabolite, was observed together with a spot corresponding to unchanged AM. Each spot corresponding to unchanged AM and the unknown metabolite was scraped from the plate, suspended in water, and centrifuged at 2000 rev/min for 5 min. The absorbance of the supernatant at 522 nm was compared. Although the chemical structure of this metabolite has not been defined, the ratio of metabolite to unchanged AM was found to be low, less than 1 per cent. Furthermore, no spot corresponding to the amines which would have been formed by fission of the azo linkage was found. This result is consistent with the finding of Radomski and Mellinger[2] that, after intrasplenic infusion, AM was almost completely excreted in the bile. These authors suggested that reduction of the azo group may be produced mainly by bacteria in the intestinal tract and that the reduction by azo reductase in the liver is of minor significance.[2]

On the other hand, it has not been reported that AS or NC is biotransformed. In the present study, we also found no evidence of biotransformation.

These results lead to the assumption that excretion of metabolites of these azo dyes is a negligible factor in the biliary excretion of these azo dyes.

When AS, AM or NC, 50 μmoles each, was administered intravenously to rats with ligated renal pedicles, 80, 74 and 50 per cent of the injected dose was excreted in the bile in 90 min respectively. From the above data, in accord with other reports,[2–4] it was found that these azo dyes are excreted in large quantities in the bile.

In order to prove that an active transport process is involved in biliary excretion, the following methods have been adopted. First, the drug was transported against a concentration gradient by an active transport mechanism. The gradient could be revealed by the estimation of liver-to-plasma and liver-to-bile concentration ratios, or the bile-to-plasma concentration ratio.[5] In the present studies, three azo dyes showed high bile-to-plasma concentration ratios of 26·4–154·3 at dose levels of 50 and 100 μmoles (Table 2), suggesting that an active transport process is involved in the biliary excretion of these dyes. Secondly, the dose of the drugs was raised to investigate whether the biliary excretion process is saturable or not.[6–9] This method is not applicable to drugs with high toxicity but only to drugs with low toxicity like the dyes used

in the present studies. As shown in Figs. 2, 3 and 4, raising the dose of AS, AM and NC resulted in saturation of the excretion rates, suggesting that an active transport process is involved. Thirdly, the effects of other drugs which have been considered to be actively secreted into bile were investigated. It might be better if these drugs closely resembled each other in chemical structure and physical and chemical properties. The three azo dyes used in the present studies are organic anions having sulfonic acid groups. In recent years, it has been proposed that various organic acids having a carboxylic acid or sulfonic acid group may be secreted into bile by a common process that has a limited transport capacity and a relatively low degree of structural specificity.[10] Probenecid is known to inhibit active hepatic secretion of numerous organic acids,[8,11] and is also known to be actively excreted into bile as a free drug and the glucuronides.[12,14] On the other hand, PPG is known to inhibit biliary excretion of conjugated bilirubin, indocyanine green[13] and succinylsulfathiazole.[9] When PPG was administered at dose levels of 15–150 μmoles to rats with ligated renal pedicles (body wt, 330–350 g), the excretion rate in bile increased to a maximum of about 9 μmoles/10 min at a dose level of 75 μmoles and remained constant on increasing the dose to 150 μmoles.* Abou-El-Makarem *et al.*[9] also found that when PPG was administered at a dose of 290 μmoles/kg of body wt, the amount excreted in the bile increased to a maximum of about 230 μmoles/kg of body wt/3 hr and remained constant on increasing the dose to 725 μmoles/kg of body wt. As shown in Figs. 5, 6 and 7, the three azo dyes were observed to be depressed by both PPG and probenecid. The inhibitory effects of PPG and probenecid on the biliary excretion of these azo dyes were marked during the first 30 min after injection, and then became less, as revealed in Figs. 5(a), 6(a) and 7(a). Such a decline of inhibitory activity is probably the result of loss of these inhibitors from the body by excretion in the bile. On the other hand, biliary excretion of PPG in this experiment was not significantly different from that of controls (Fig. 8). These results suggest that these azo dyes and inhibitors may share at least a common excretory process and that the affinity of PPG may be higher than that of these dyes for the common process.

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