Br. J. Pharmac. (1971), **41**, 140–153.

Studies on the inhibition of clearance of organic dyes by Saramycetin

D. A. COONEY, R. D. DAVIS AND E. R. HOMAN

Laboratory of Toxicology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

N. RAKIETEN

South Shore Analytical and Research Laboratory Inc., Islip, New York, USA

U. SCHAEPPI

Mason Research Institute Inc., Worcester, Massachusetts, USA

G. VANATTA

Microbiological Associates Inc., Bethesda, Maryland, USA

Summary

1. Saramycetin, a polypeptide antifungal antibiotic has been found to retard the clearance of sulphobromophthalein (BSP) in man. An explanation for this observation was sought in several lower species.

2. Doses of Saramycetin without effect on the other standard tests of hepatic function or on hepatic morphology profoundly altered the disposition of BSP and several other dyes in mice and dogs.

3. Saramycetin strongly inhibited the hepatic enzyme which conjugates BSP to reduced glutathione, provoked a regurgitation of BSP from the liver into the bloodstream, and was anticholeretic in the dog.

4. These diverse actions of Saramycetin may, in concert, explain the altered clearance of BSP. It is suggested that low doses of Saramycetin exert a pharmacological effect on certain hepatic excretory processes, whereas high doses are toxic.

Introduction

Saramycetin (NSC-100,844) is a polypeptide antifungal antibiotic produced by *Streptomyces saraceticus* (Baudet & Cherbuliez, 1964). During early clinical trials of the drug in patients with disseminated mycoses, delayed clearance of the dye sulphobromophthalein (BSP) was invariably observed (Andriole, Utz & Sabesin, 1961). Because the elimination of BSP constitutes one of the most sensitive clinical tests of hepatic function, this finding was felt to be a serious deterrent to further clinical trials of Saramycetin, despite its efficacy. The basis for the drug induced retention of BSP was therefore studied.

Methods

Saramycetin was obtained from the Squibb Institute for Medical Research. BSP purchased from the J. P. Baker Company, Muirkirk, Maryland, was found to be paper chromatographically pure in a descending system of *n*-butanol : acetic acid : water, 4 : 1 : 5 and 4 : 1 : 1.

Fluorescein-sodium and taurocholate-sodium were purchased from Calbiochem ; Rose Bengal (81% total dye content) from Matheson, Coleman and Bell, East Rutherford, N.J. ; phenolsulphophthalein (0·006 g/ml, USP) from Harvey Laboratories ; reduced glutathione from K. and K. Laboratories, Plainview, N.Y. ; and Pentobarbitone-sodium (Diabutal) from Diamond Labs., Inc., Des Moines, Iowa.

Swiss mice (Texas Inbred Mice Co., Houston, Texas) on an *ad libitum* diet of Purina laboratory chow and water, were used for lethality and distribution studies ; beagle dogs were used for the subacute toxicity studies. Adult male albino guinea-pigs were utilized for anaphylaxis tests, and albino rabbits for production of antisera against Saramycetin ; both species were purchased from Roma Rabbitry, Mt. Airy, Maryland.

Passive haemagglutination was accompanied by incubating saline washed sheep erythrocytes (Suburban Serum Co., Silver Spring, Maryland) coated in 10^{-3}M Saramycetin at 37° C 1 h and given a single saline wash. Anti-Saramycetin antiserum was raised in a rabbit by repeated intramuscular injections of the antibiotic in complete Freund's adjuvant. The antibody was labelled with fluorescein on celite (Rinderknecht, 1960) followed by dialysis.

The spectrophotometric method of Goldstein & Combes (1966) was used for determination of the glutathione-BSP conjugating enzyme. The BSP-glutathione conjugate was separated from unchanged BSP by the method of Maggio & Fujimoto (1966) in a 20-tube countercurrent apparatus obtained from the E.C. Apparatus Co., Philadelphia, Pa., using as phases 1 N-HCl : diethyl ether : *n*-butanol, 4 : 3 : 1.

For the determination of distribution-patterns of BSP after its intravenous injection, organs were homogenized in distilled water ; the resulting suspensions were adjusted to 100 ml and centrifuged at 30,000 *g* ; aliquots of the supernatants were either diluted further (in the case of liver) or read directly in the Beckman DB spectrophotometer at 575 nm before and after alkalinization to pH 10·0 with 2 N-NaOH. In the studies on murine choleresis, Pasteur pipettes drawn to a fine point and alkalinized with a film of NaOH, were used to empty the gallbladder by capillary action. Assays of Saramycetin in cerebrospinal fluid and serum were conducted against *Paecilomyces varioti* according to the methodology of Grunberg, Berger & Titsworth (1961). Choleretic studies in the dog were carried out in unanaesthetized beagles whose neuraxis had been transected at the level of the midbrain. Polyethylene tubing (Intramedic PE 205, I.D. 1·57 mm) was used to cannulate the common bile duct.

The removal of indocyanine green (ICG ; Cardio-Green, Hynson, Wescott and Dunning, Baltimore, Maryland) was measured in female mice (NIH CAFl hybrid strain) anaesthetized with intraperitoneal pentobarbitone, 100 mg/kg. Additional doses of anaesthetic, 20 mg/kg, were administered as necessary. Mice were maintained on an externally warmed surface and the rectal temperature monitored with a thermistor probe YS1 Tele-Thermometer. The bile duct was catheterized with a polyethylene tube (Intramedic PE 20, I.D. 0·381 mm) tapered with gentle heat and

stretching. Bile was collected in 20 μl capillary pipettes for measured intervals and the flow calculated by measuring the length of a 20 lambda pipette filled. Bile samples were diluted and their absorbance measured at 787 nm using a Beckman DB-G spectrophotometer with a red-sensitive R136 phototube.

ICG at a concentration of 5 mg/ml in distilled water was administered intravenously through a tail vein cannula made from a 30 gauge dental hypodermic needle connected to a syringe by a length of PE 10, O.D. 0·279 mm polyethylene tubing. Saramycetin in physiological saline was administered intravenously through the same cannula.

Measurements of the concentration of ICG in blood were made with a dichromatic earpiece densitometer (Model XE-302, Waters Instrument Company, Rochester, Minnesota). The earpiece was fitted with an adapter which permitted the insertion of a shaved forelimb of the mouse for the continuous *in vivo* measurement of dye concentration. The resulting electrical signal was recorded on a Beckman Linear-Log Recorder operated in the linear mode.

Results

Effects on BSP clearance

Species specificity

Saramycetin significantly retarded the clearance of BSP in the dog and mouse (Fig. 1). The phenomenon has also been demonstrated in rats and rabbits. The doses of antibiotic just capable of producing this effect are listed in Table 1 ; in the dog, as little as 0·5 mg/kg (10 mg/M^2, $2{\cdot}5 \times 10^{-4}$ mmol/kg) of antibiotic impaired the removal of BSP.

Toxicity of Saramycetin

Several liver function tests (Table 2) were performed in the beagle at the threshold dose ; no derangements of liver function, apart from impaired clearance of

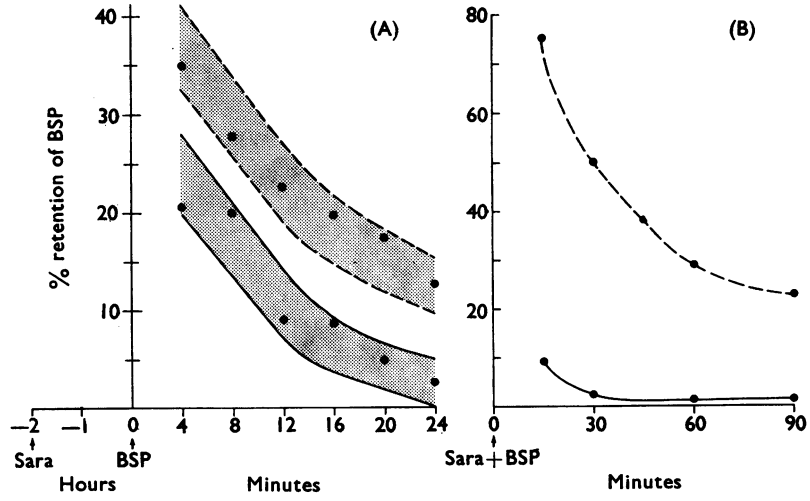

FIG. 1. Influence of Saramycetin (Sara) on the clearance of BSP in mice (A) and the beagle dog (B). (A), Swiss mice received BSP, 100 mg/kg intravenously (——) or BSP 100 mg/kg intravenously plus Saramycetin 200 mg/kg subcutaneously (– – –) at the times indicated. Each point represents the mean % retention of twenty mice. The range of determinations is indicated by stippling. (B), In the morning the dog received BSP 5 mg/kg intravenously (——). In the afternoon the dog received BSP 5 mg/kg along with Saramycetin 8 mg/kg (– – –).

TABLE 1. *Threshold doses of Saramycetin causing BSP retention*

Species and no. animals	Dose of BSP mg/kg	Dose of BSP mg/M²	Minimum dose of Saramycetin which retards BSP clearance mg/kg	Minimum dose of Saramycetin which retards BSP clearance mg/M²	Interval between injection of Saramycetin and subsequent dose of BSP	% BSP retained after 30 min: Before Saramycetin	% BSP retained after 30 min: After Saramycetin
Dog (2)	5	100	0·5 i.v.	10	15 min	4	10
Dog (2)	15	300	0·15 i.v.	3	5 min	10	89
Mouse (5)	100	300	50 s.c.	150	2 h	3	10
Mouse (5)	100	300	5 i.v.	15	0 min	3	10

TABLE 2. *Chronic and acute toxicity of Saramycetin (NSC-100844) given intravenously to beagle dogs*

Dose mg/kg × days		Overt signs†	Haematology	Nadir†	Blood chemistry†	Pathology†
One beagle killed day 56 (well)	128 × 9	Cholinergic syndrome (emesis, defaecation, urination, prostration, convulsive movements, muscle tremors, salivation tearing, blinking, dyspnoea, bradycardia, hyperventilation); haematuria; 21% weight loss	Haematocrit ↓ leucocytosis	(31%) ($22{\cdot}8 \times 10^3$)	SGOT ↑ (220 U/ml) SGPT ↑ (240 U/ml)† BSP retention (63·3%) Sed. rate ↑ (36 mm/h)	Liver: reactive hyperplasia Pancreas: chronic fibrosing pancreatitis Salivary gland: chronic sialadenitis
One beagle killed day 13 (moribund)	64 × 12	Cholinergic syndrome, muscle tremors, disorientation, emesis, anorexia, ataxia, 14% weight loss	Haematocrit ↓ thrombocytopenia	(29%) ($0{\cdot}9 \times 10^5$)	BSP retention (53·6%) Sed. rate ↑ (120 mm/h)	Liver: inspissated bile in large biliary radicles; central vein phlebitis; intracellular and intracanalicular cholestasis; cytoplasmic vacuolization of hepatocyte
Six beagles killed days 16 and 36 (well)	32 × 14	6% weight loss	Haematocrit ↓	(30%)	BSP retention (36%) [6/6]† SGOT ↑ (77 U/ml) [1/6] SGPT ↑ (55 U/ml.) [1/6]	Liver: reactive hepatitis [1/6] Liver: reactive hyperplasia [1/6] Lung: focal chronic bronchitis and tracheitis [1/6]
Two beagles killed days 16 and 29 (well)	16 × 14	Hyperthermia [1/2]	Within normal limits		BSP retention (25%) [2/2]	Liver: mild reactive hepatitis [2/2] Injection site: mild phlebosclerosis [1/2]

U = Sigma Frankel units; † number of animals exhibiting toxicity/number of test animals given in brackets.

BSP, were detectable. Unequivocal hepatotoxicity could, however, be produced by Saramycetin in all species at doses above those which retarded the clearance of BSP.

Pentobarbitone sleeping times were measured in Swiss mice treated with 100 mg/kg of the antibiotic subcutaneously daily for 10 days. A significant prolongation of the sleeping time of Saramycetin treated animals was observed (Fig. 2).

The immunogenicity of Saramycetin could be demonstrated in two ways: by its provocation of true non-fatal anaphylaxis in ten out of ten guinea-pigs sensitized in the classical manner and by the production of rising haemagglutinin titres of antibody (1:2→1:128) in mice, as demonstrated with passively coated ovine erythrocytes. The influence of the immune state on the altered removal of dyes which Saramycetin causes in normal subjects, is under study.

Timing of the phenomenon

In dogs Saramycetin retarded the clearance of BSP whether the antibiotic was given before, simultaneously, or after the injection of dye. After giving 8 mg/kg for 14 days, impairment of the clearance of BSP was demonstrable for 24 h following the final dose; after 16 mg/kg for 14 days, abnormal clearance persisted for 48 h; and after 32 mg/kg for 14 days BSP retention was detectable for at least 5 days after the last dose. Nevertheless, on the day following the last injection of Saramycetin at 32 mg/kg, no antibiotic could be demonstrated in the plasma by antifungal assay against *P. varioti*; nor were histopathological abnormalities de-

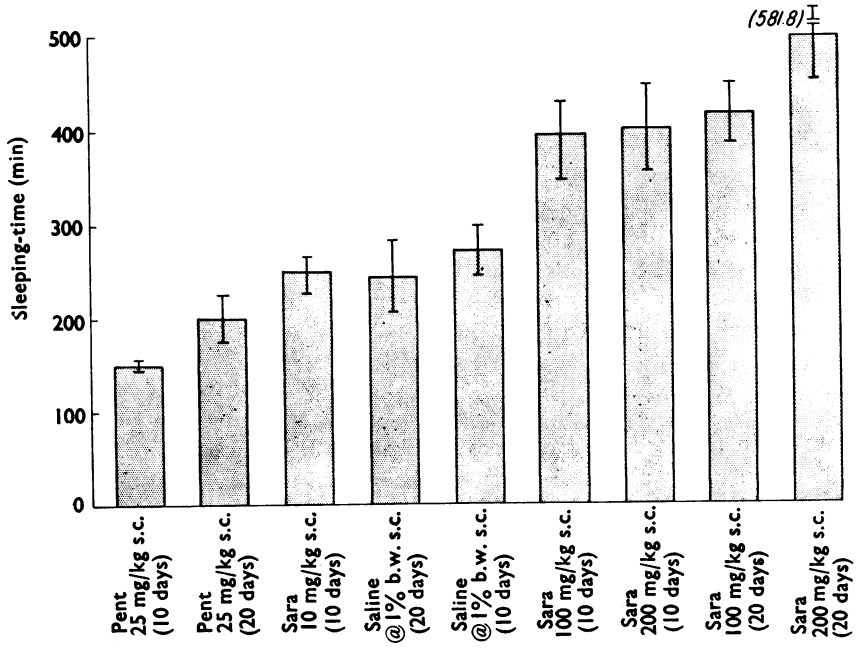

FIG. 2. Influence of Saramycetin on the sleeping time of Swiss mice (eight to twelve mice/group) given an intraperitoneal challenge dose of pentobarbitone, 80 mg/kg. The animals were pretreated daily with pentobarbitone (Pent), saline or Saramycetin (Sara) given subcutaneously for the number of days indicated. The range of sleeping times has been indicated by bars. b.w.=body weight.

tected in the liver of a majority (five out of six) of the dogs (Table 2). Firm binding of Saramycetin is a possible explanation for this residual effect. However, attempts to demonstrate Saramycetin bound to the hepatocytes of these animals with fluorescein-labelled anti-Saramycetin antisera have failed.

Saramycetin significantly delayed the elimination of BSP when it was administered intravenously shortly before, or at the time of injection of dye. Moreover in mice, even when the antibiotic was given after dye, further clearance of BSP was halted for at least 20 minutes.

Variation of the dose of BSP

In an attempt to explore further the interaction of BSP with Saramycetin, the role of varying doses of dye was studied. It was found that 0·15 mg/kg of Saramycetin, a dose with little effect on the clearance of a standard dose of BSP (5 mg/kg) in the dog, would retard the elimination of 15 mg/kg of dye. Thus 60 min after a triple dose of dye, 35% retention of BSP was observed in the dog pretreated with Saramycetin as opposed to 3% retention in the same dog during the control period.

Influence of routes of administration of Saramycetin

Saramycetin given intravenously or subcutaneously impaired the removal of BSP (Table 1), but oral administration of the antibiotic, 200 mg/kg, in mice failed to alter dye clearance.

Disposition of Saramycetin in body fluids

The half-time of Saramycetin in the plasma of two beagles following an intravenous dose of 8 mg/kg followed a biphasic disappearance curve with a very rapid initial clearance ($T_{1/2}$=5 min) followed by a slow and shallow limb ($T_{1/2}$=1 h). The concentration of the antibiotic in plasma was maximal one minute after dosing (7 μg/ml) and rapidly declined thereafter according to the rates of clearance given above. Small but significant quantities of Saramycetin (250 μg/l.) have been detected in the urine for 75 min after an intravenous dose of 2 mg/kg, although no plasma levels were measurable over this period. Thereafter, the urinary excretion fell to zero. No Saramycetin could be detected in the cerebrospinal fluid at 20 and 100 min after dosing dogs with 64 mg/kg.

Interaction of Saramycetin with other organic dyes

Saramycetin retarded the clearance not only of BSP, but also of the fluorescein-derivative, Rose Bengal, a dye which has been used extensively in the evaluation of hepatic function (Stowe, Delprat & Weeks, 1933). In one beagle the 6 min clearance of Rose Bengal decreased between 30% and 63% when compared with the animal's pretreatment value ; 5 days later the clearance had returned to normal.

Rose Bengal, BSP and fluorescein have similar molecular skeletons and are excreted principally by the liver into the bile. The removal of indocyanine green (ICG), a dye whose structure is less like that of BSP, was also retarded by Saramycetin. Half times of ICG in the plasma during the two control periods were 1·24 and 1·33 min, but after intravenous administration of 25 mg/kg of Saramycetin

rose to 17·5 minutes. In some preparations there was, in fact, a small subsequent rise of circulating ICG possibly representing displacement of dye from binding sites in the liver. The removal of a subsequent dose of ICG following Saramycetin was even more dramatically retarded ($T_{1/2}$=21 min). On the other hand, biliary elimination of ICG apparently was not interrupted by Saramycetin, although subsequent injections of ICG failed to elevate the concentration of dye in the bile.

It is interesting that in the dog the timed renal excretion of phenolsulphonphthalein, an organic dye structurally related to BSP, but excreted principally by the kidneys, was unaffected by Saramycetin given at doses (8 mg/kg)/day × 14, intravenously) which markedly retarded the clearance of BSP.

Mechanism of action of Saramycetin

Transport of BSP in the plasma

Many dyes are bound by plasma proteins and this binding could play an important role in the clearance of BSP. Since BSP is bound principally to albumin, it was reasonable to suppose that Saramycetin, by enhancing the affinity of the protein for the dye, could retard its elimination. However, in dialysis experiments with foetal calf serum and human plasma, *in vitro*, Saramycetin failed to promote any enhanced binding of dye to protein or to alter the dialysis equilibrium unless the antibotic was present in very high (1%) concentrations. In this case, Saramycetin, a protein itself, was able reversibly to bind BSP. It should be stressed that this binding is unlikely to explain the effects of Saramycetin on BSP uptake since the concentration of the antibiotic used *in vitro* was several orders of magnitude greater than the highest level measured in plasma. In addition, cellulose-acetate electrophoresis of plasma from Saramycetin pretreated mice that had received BSP 5 min before sampling, failed to reveal any differences from controls in the distribution of dye on the plasma proteins that could not be explained by the higher levels of BSP present in the pretreated animals.

Entry of BSP into the hepatocyte

It is possible that Saramycetin might retard the entry of BSP into the hepatocyte. In order to explore this possibility, Swiss mice were pretreated with Saramycetin, challenged with BSP 2 h later, and at several time periods thereafter killed and the

TABLE 3. *Influence of Saramycetin on hepatic, renal and intestinal levels of BSP at different times after dosing*

Controls			
BSP content of liver (μg)		BSP content of small intestine (μg)	BSP content of kidney (μg)
5 min	10 min	30 min	60 min
769±60	1198±36	510±63	10·85±4
Saramycetin pretreated			
435·5±25·76	577±14	420±32	20·2±5

Two hours before the intravenous injection of BSP, groups of ten Swiss mice were pretreated with Saramycetin, 200 mg/kg, subcutaneously. Controls received saline. At 5, 10, 30 and 60 min after the injection of BSP, 4 mg intravenously, the animals were killed by cervical dislocation and the organs listed in the table removed for homogenization or lavage; BSP content was estimated colorimetrically as described in **Methods**. The figures represent the mean values for ten mice ± the standard error.

BSP measured in the small intestine, liver and kidneys (Table 3). It will be seen that the intestinal levels of dye are only lowered by 20% after pretreatment with Saramycetin whereas the hepatic content of BSP is halved.

Histochemical studies with Rose Bengal, a moderately strong red fluorochrome, have qualitatively confirmed these results with BSP. Swiss mice pretreated either with saline or with Saramycetin, 100 mg/kg intravenously received 2 h later, Rose Bengal, 100 mg/kg intravenously (Grafflin & Bagley, 1952). Whereas after saline, the hepatocytes fluoresced brightly, especially at the cell margins and in a few of the biliary tracts, hepatocytes from animals receiving Saramycetin exhibited only a diffuse, dim cytoplasmic fluorescence.

The opposite effect was seen in the kidneys. Levels of BSP in the renal homogenates of Saramycetin treated animals were higher than in their saline treated counterparts. In the face of the persistently high plasma levels of dye which

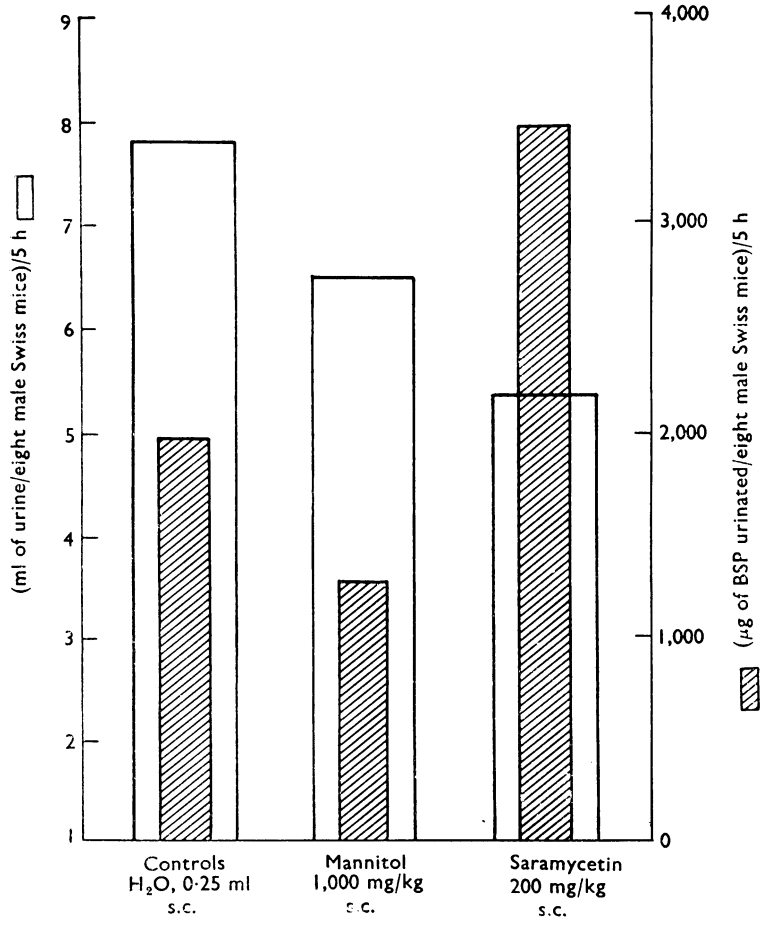

FIG. 3. Influence of Saramycetin on the urinary excretion of BSP. Eight Swiss mice per group were housed in metabolism cages and their urine collected in a dry-ice cooled vessel. One hour before the injection of BSP, the control group received 0·25 ml of water, subcutaneously; the diuresis controls received mannitol 1000 mg/kg subcutaneously, and the experimental animals received 200 mg/kg of Saramycetin subcutaneously. Urine collection was continued for 5 h after the intravenous injection of BSP, 100 mg/kg; dye-content was measured colorimetrically at 575 nm in a Beckman DB spectrophotometer.

Saramycetin can cause, it would seem reasonable to postulate that the kidneys assume the excretory function normally accomplished by the liver. In keeping with this observation is the pattern of urinary excretion of BSP in controls and Saramycetin pretreated mice. This pattern is illustrated in Fig. 3.

It will be seen that the antibiotic promoted the renal excretion of BSP in these circumstances whereas mannitol, used as a diuretic, did not.

Conjugation of BSP with glutathione

BSP is excreted unchanged and as a thioether conjugate of glutathione (Combes & Stakelum, 1960). There is evidence that this conjugate is more promptly cleared than unchanged BSP (Combes, 1965). If Saramycetin depressed this conjugation, the net removal of dye would be retarded. Saramycetin at 5×10^{-7}M was found to inhibit the conjugation of BSP to glutathione *in vitro*. Analysis of the kinetics (Fig. 4) indicates that this inhibition is of the non-competitive type. However, when livers from animals pretreated *in vivo* with 200 mg/kg of Saramycetin were used as the source of conjugating enzyme, only marginal inhibition of conjugation was detectable.

Despite the relatively modest inhibition of the BSP-glutathione conjugating enzyme by Saramycetin injected *in vivo*, rather marked depression of the absolute level of the BSP-glutathione conjugate itself (to about 50% of control) was observed in the livers of the pretreated mice. Countercurrent distribution was used to separate the conjugate from unchanged BSP (Maggio & Fujimoto, 1966). The depressed level of the BSP-glutathione conjugate possibly stems as much from the lower levels ($\sim$45% of control) of intracellular dye available for enzymatic conjugation as from inhibition of the conjugation itself.

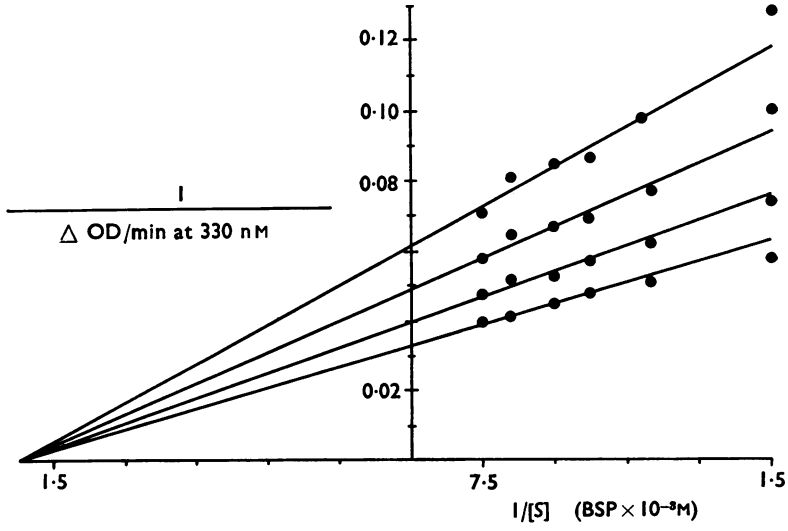

FIG. 4. **Kinetics of the inhibition by Saramycetin of the BSP-glutathione conjugating enzyme.** The spectrophotometric assay of Goldstein & Combes (1966) was used. The lowest line represents the uninhibited reaction; the line above it represents the reaction conducted in the presence of Saramycetin, 5×10^{-7}M; the third line from the bottom represents the reaction conducted in the presence of Saramycetin, $1{\cdot}25 \times 10^{-6}$M; and the top line represents the reaction conducted in the presence of Saramycetin, $2{\cdot}5 \times 10^{-6}$M.

Excretion of BSP into the biliary passages

It is conceivable that Saramycetin might prevent the exit of BSP from the hepatocyte. In order to explore this possibility, the cholecystic appearance times of two dyestuffs, BSP and its fluorescent congener, fluorescein were measured in mice by chromocholoscopy (Baiotti & Abrate, 1966). The dyes were injected intravenously into animals pretreated either with saline or Saramycetin. A rapid laparotomy was performed, and the contents of the gallbladder were aspirated with an alkalinized Pasteur pipette drawn to a very fine point. Only bile that was unequivocally purple-blue, in the case of BSP, or brilliantly fluorescent under ultraviolet light, in the case of fluorescein-sodium, was given a positive score. The results of this study are given in Fig. 5. Saramycetin significantly delayed the appearance of both dyes in gallbladder bile.

Although bile flow in mice given repeated doses of ICG did not change after the administration of Saramycetin, an abrupt but transitory anticholeretic effect was observed when the antibiotic alone was given intravenously to beagles. Taurocholic acid was still able to produce a significant choleresis after the effect of Saramycetin had passed, thus insuring the viability of the whole hepatobiliary apparatus in these studies. Inasmuch as Klaasen & Plaa (1967) have stressed the importance of bile

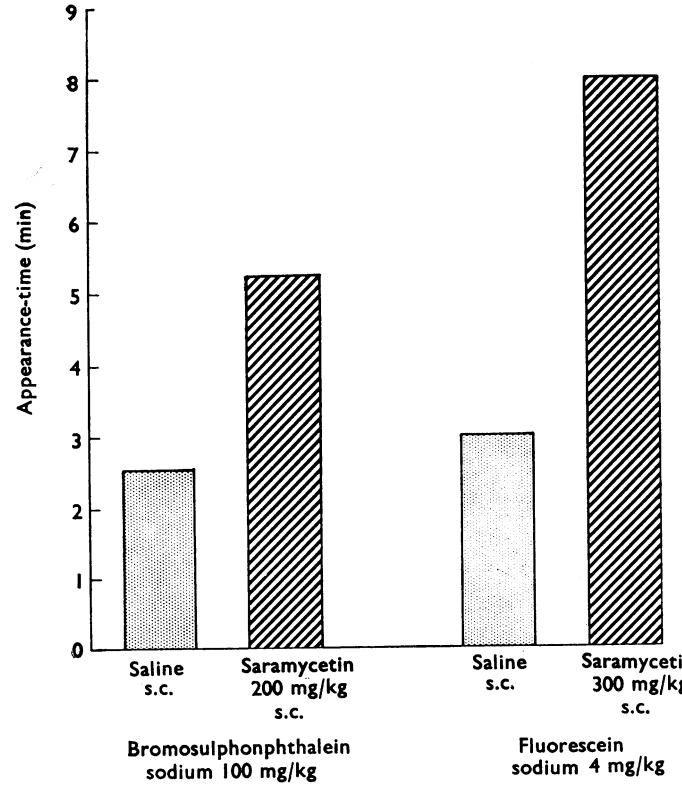

FIG. 5. Appearance-times of BSP and fluorescein in the gallbladder of the mouse. Two hours before the intravenous injection of BSP, 100 mg/kg, or fluorescein-Na, 4 mg/kg, six Swiss mice were pretreated with subcutaneous Saramycetin, 200 and 300 mg/kg respectively. At 30 s intervals after injection of the dyes, the gallbladders were quickly exposed. Bile was sampled as described in **Methods**. The mean appearance times are indicated by bars. Within 30 s of these mean times all six animals in each group had dye in the gallbladder.

flow in the net clearance of BSP and congeneric dyes, this anticholeretic action of Saramycetin may be significant in dogs.

Reflux of BSP

Another possible explanation of the influence of Saramycetin on the clearance of BSP is that the antibiotic can dislodge BSP from intracellular hepatic sites. To explore this hypothesis, BSP was given intravenously to beagles, and its elimination allowed to proceed. When 90% clearance had been achieved, Saramycetin was injected into a contralateral saphenous vein. A significant regurgitation of dye was observed, followed by a prolonged elimination phase similar to that which was observed when Saramycetin was given as pretreatment (Fig. 6).

Enhanced enterohepatic circulation

It is possible that Saramycetin may augment the ordinarily slight enterohepatic circulation of BSP which Lorber, Oppenheimer, Shay, Lynch & Siplet (1953) have

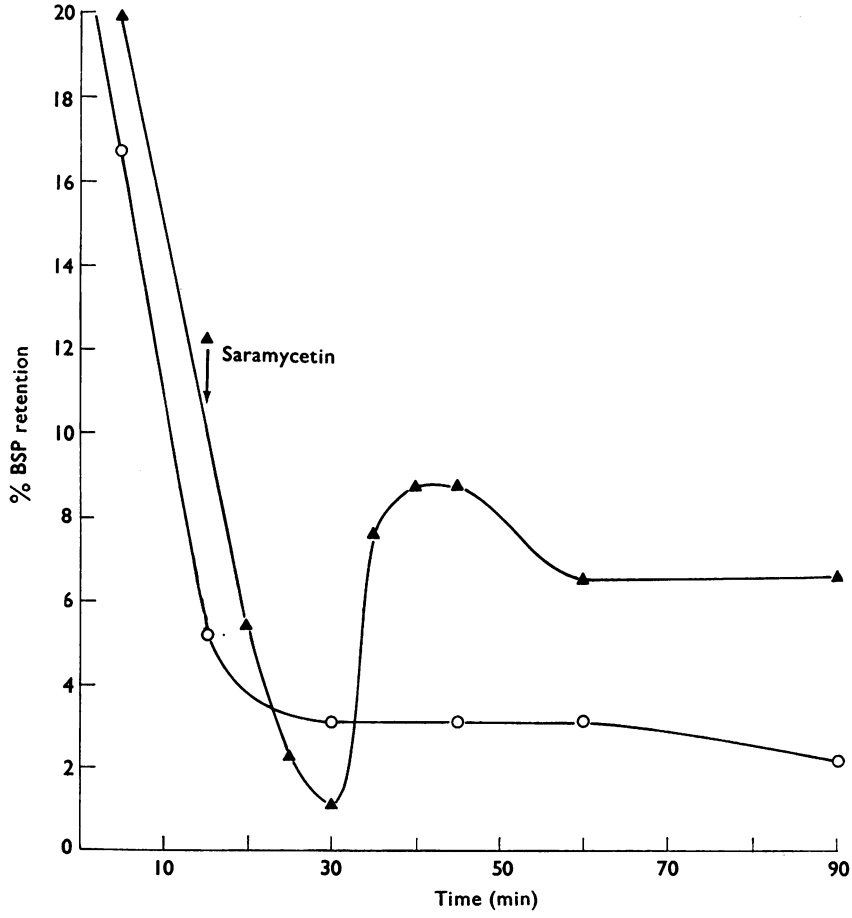

FIG. 6. Regurgitation of BSP, caused by Saramycetin, in the dog. Fifteen minutes after the intravenous injection of BSP, 5 mg/kg, Saramycetin was injected intravenously at a dose of 32 mg/kg (▲). The BSP content of plasma samples withdrawn at the time periods shown, was measured colorimetrically. The beagle served as his own control on the day preceding the Saramycetin study (○).

documented. If this were in fact the case, intestinal levels of dye would be expected to be lowered as a consequence of enhanced recirculation of dye. As has been mentioned, however, luminal dye levels in the small intestine of normal and Saramycetin pretreated mice given intravenous BSP were not significantly different (Table 3). No direct duodenal instillations of dye were attempted however in the present study.

Discussion

When a drug interacts with its receptor without damage, its effects can be said to be pharmacological; when the interaction is detrimental the effects can be said to be toxic. In the case of Saramycetin, the phenomenon of delayed clearance of BSP should be considered a pharmacological property of the drug. Thus, while the antibiotic has been shown to be hepatotoxic at high repeated doses, effects on the clearance and conjugation of BSP are detectable even at low non-toxic single doses. Moreover, when the influence of pretreatment with Saramycetin on the pentobarbitone-metabolizing enzymes of mouse liver was studied, prolongation of the sleeping times of mice was also seen at doses which inflicted no morphological damage to the liver. Thus the antibiotic appeared to inhibit the induction of hepatic drug-metabolizing enzymes in the mouse in non-toxic doses.

In seeking an explanation for the altered clearance of dyes which Saramycetin causes, several possible sites of action were investigated. Although deranged clearance of several dyes has been demonstrated in cases of congenital analbuminaemia (Bennhold, 1966), it was not possible to demonstrate that Saramycetin altered the ability of albumin to bind BSP. A second potential site of action of Saramycetin is the hepatocyte membrane on the capillary side. Since the antibiotic significantly reduces the intracellular concentration of BSP and other dyes, it is likely that Saramycetin impedes the uptake of these organic dyes. The alternate explanation, that Saramycetin accelerates the excretion of these dyes, thereby lowering their intrahepatic concentrations, is considered unlikely in the light of results discussed below. In the third place it is possible that Saramycetin acts by impairing the conjugation of BSP with glutathione by the intracellular enzymatic system of the hepatocyte. In fact, Saramycetin has been found to be a very powerful inhibitor of this enzyme. The fact that Saramycetin could even provoke a regurgitation of BSP which was undergoing clearance suggests that Saramycetin might have a greater affinity than BSP itself for some receptor within the hepatocytes. In view of the powerful inhibition of the BSP-glutathione conjugating enzyme by Saramycetin, it is possible that this protein is the receptor in question. However, inhibition by Saramycetin of the enzymatic coupling of BSP to glutathione cannot wholly explain the phenomenon which prompted this study, since neither Rose Bengal, ICG nor fluorescein are excreted as glutathionates.

In order to assess the interaction of Saramycetin with the canalicular side of the hepatocyte, chromocholoscopy was employed. The finding of significantly delayed biliary excretion rates of dyes in animals pretreated with Saramycetin is rendered somewhat ambiguous by the experimental techniques used, by the tortuous and compartmentalized anatomy of the biliary apparatus, and by the apparent species differences in bile flow that were uncovered. Nevertheless, while sampling from the fundus of the gallbladder may not have permitted exact timing, the results do

point to an inhibition of excretion of dyes by Saramycetin. Moreover, since the antibiotic was not anticholeretic in the mouse (in contrast to the dog), these studies with murine gallbladder bile are felt to be valid indices of inhibition of excretion. On the basis of these studies, then, it is likely that Saramycetin interacts with both the capillary and canalicular faces of the hepatocyte, preventing the free passage of several organic dyes; and that in the case of BSP, those dye molecules which do enter the hepatic cytoplasm are conjugated to glutathione at a diminished rate in the presence of the drug.

This work was supported in part by contract Nos. PH43-64-932, PH43-65-51 and PH43-68-1283.

REFERENCES

ANDRIOLE, V. T., UTZ, J. P. & SABESIN, S. M. (1961). Altered sulfobromophthalein metabolism in man induced by the antifungal agent X-5079C. *Am. Rev. Resp. Dis.*, **84**, No. 4, 538–548.

BAIOTTI, G. & ABRATE, M. (1966). La chromocoloscopia alla bromosulfaleina nella diagnosi differenziale degli itteri occlusivi intra ed extraepatici. *Minerva Med., Paris*, **57**, 3038–3041.

BAUDET, P. & CHERBULIEZ, E. (1964). Sur la saramycétin (=X-5079C), polypeptide antibiotique de Streptomyces saraceticus. *Helv. chim. acta.*, **47**, Fasc. 2, 661–683.

BENNHOLD, H. (1966). Transport function of the serum proteins: historical review and report on recent investigations on the transport of dyestuffs and of iron. *Transport Function of Plasma Proteins*, pp. 1–21, eds. Desgrez, P. & Traverse, P. M., Amsterdam: Elsevier Publishing Co.

COMBES, B. (1965). The importance of conjugation with glutathione for sulfobromophthalein sodium (BSP) transfer from blood to bile. *J. clin. Invest.*, **44**, 1214–1224.

COMBES, B. & STAKELUM, G. S. (1960). Conjugation of sulfobromophthalein sodium with glutathione in thioether linkage by the rat. *J. clin. Invest.*, **39**, 1214–1222.

GOLDSTEIN, J. & COMBES, B. (1966). Spectrophotometric assay of the liver enzyme that catalyzes sulfobromophthalein-glutathione conjugation. *J. Lab clin. Med.*, **67**, 863–872.

GRAFFLIN, A. L. & BAGLEY, E. H. (1952). Studies of hepatic structures and function by fluorescence microscopy. *Johns Hopkins Hosp. Bull.*, **90**, 395–435.

GRUNBERG, E., BERGER, J. & TITSWORTH, E. (1961). Chemotherapeutic studies on a new antifungal agent, X-5079C, effective against systemic mycoses. *Am. Rev. resp. Dis.*, **84**, No. 4, 504–506.

KLAASEN, C. D. & PLAA, G. L. (1967). Determination of sulfobromophthalein storage and excretory rate in small animals. *J. appl. Physiol.*, **22**, (6), 1151–1155.

LORBER, S., OPPENHEIMER, M., SHAY, H., LYNCH, P. & SIPLET, H. (1953). Enterophepatic circulation of bromosulphalein: intraduodenal, intraportal and intravenous dye administration in dogs. *Am. J. Physiol.*, **173**, 259–264.

MAGGIO, M. B. & FUJIMOTO, J. M. (1966). Effect of carbon tetrachloride on distribution of sulfobromophthalein in plasma and liver of mice. *Tox. Appl. Pharmac.*, **9** (2), 309–318.

RINDERKNECHT, H. (1960). A new technique for the fluorescent labelling of proteins. *Experientia*, **16**, 430.

STOWE, W. P., DELPRAT, G. D., & WEEKS, A. (1933). The rose bengal test of liver function. *Am. J. clin. Path.*, **3**, 55–60.

(*Received January*, 20, 1970)