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The effect of salicylate on the biliary excretion of ^{14}C-bishydroxycoumarin in rat

H. S. BUTTAR, B. B. COLDWELL AND B. H. THOMAS

Drug Research Laboratories, Health Protection Branch, Department of National Health and Welfare, Ottawa, Canada

Summary

1. The effect of intravenous sodium salicylate on the biliary excretion of ^{14}C-bishydroxycoumarin (^{14}C-BHC) was studied in rats.

2. Salicylate (88·9 mg/kg) increased the biliary excretion of ^{14}C-radioactivity from a control value of 12·3 ± 2·7% to 29·3 ± 2·5% of the administered dose in 6 h after injection.

3. During the 6 h period, 11% of the dose of radioactivity underwent biliary recycling in the salicylate-treated rats compared to only 6% in the absence of salicylate.

4. About 15·3% of the radioactivity excreted in the bile of rats given BHC alone was detected as unchanged BHC, 8·6% was present as BHC-conjugate, conjugates of BHC metabolites accounted for 30·9%, and the remainder consisted of unidentified metabolites.

5. Salicylate treatment did not significantly alter the excretory pattern of unchanged BHC and its metabolites.

Introduction

We have shown that the prothrombin time of blood taken from rats on a regimen of bishydroxycoumarin (BHC) 18 h after treatment with salicylate was reduced (Coldwell & Zawidzka, 1968 ; Zawidzka & Coldwell, 1970). The blood level of ^{14}C-BHC was depressed and its half-life shortened by acetylsalicylic acid and sodium salicylate. In addition, the liver concentration of ^{14}C was 2-fold greater in the salicylate-treated rats (Thomas, Coldwell, Buttar & Zeitz, 1973). Since bile secretion is stimulated by salicylates (Okada, 1915 ; Schmidt, Beazell, Atkinson & Ivy, 1938), the possibility that the rapid decay of radioactivity from the blood of salicylate-treated rats was due to the enhanced excretion of BHC and its metabolites into the bile was investigated.

Methods

Drug solutions

A 1% (w/v) aqueous solution of BHC (Charles E. Frosst Co.), pH 12·8, was prepared (Lucas, 1967). Solutions containing ^{14}C-BHC (methylene-^{14}C) (NEN Corp., Boston, Mass.) were made so that the final solution had a specific activity of 0·67 μCi/mg. Sodium salicylate solution (5% w/v) was prepared in 0·9% w/v NaCl solution (saline).

Animals and treatment

Male Albino rats (Wistar, 275–355 g) with unrestricted access to purina rat chow and water and acclimatized for seven days to the laboratory environment were pretreated by i.p. injection of BHC, 20 mg/kg on the first day and 15 mg/kg on the next day.

A polyethylene tube (PX-011) was inserted into the common bile duct under ether. The femoral vein was similarly cannulated and connected to a disposable needle (No. 26G). In studies of the enterohepatic circulation of BHC, venous cannulation and laparatomy were performed but the bile duct was left intact. Rectal temperature was measured with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) and the body temperature maintained between 36° and 36·7° C.

One hour after surgery on the third day, one group of rats was given ^{14}C-BHC (15 mg/kg, i.v.); another group received sodium salicylate (88·9 mg/kg, i.v.) immediately after the anti-coagulant. A few animals were given salicylate only. Previous studies showed that these drug dosages lengthened the one-stage prothrombin time from the normal value of about 13 s to about 22 s (Coldwell & Zawidzka, 1968), and that a single dose of salicylate was as effective in decreasing the prothrombin time as daily dosing for 5 weeks (Zawidzka, Coldwell & Grice, 1972).

Collection of body fluids and tissues

Immediately following surgery control bile samples were collected for 1 hour. A bile flow of 0·5 ml/h was considered satisfactory. Following injection of the drugs bile was collected for 6 hours. Fluid lost was replaced hourly by i.v. administration of saline. The samples were measured and stored at 4° C and analysed within 2 weeks.

Radioactivity profiles were determined in duplicate 20 μl samples of tail blood and bile taken at intervals up to 6 h following injection of the drugs.

At 6 h the livers were removed, blotted and weighed and duplicate samples (50–100 mg) analysed for radioactivity. Urine from the bladder was removed with a syringe, and duplicate 20 μl aliquots taken for estimation of radioactivity. The intestine was removed after being tied off at the pylorus and the rectum, and washed thoroughly in cold tap water. The contents were weighed, homogenized with distilled water (1:9) and duplicate 50 μl aliquots were taken for radioactivity analysis.

Determination of radioactivity

The total concentration of ^{14}C-BHC and its metabolites was determined in duplicate 20 μl samples of blood, bile, and urine, 50 μl of faecal homogenate or 50–100 mg liver added to 1 ml of Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) in scintillation counter vials. Digestion was completed by shaking overnight at room temperature. Toluene based scintillation fluid (10 ml) containing 0·6% w/v 2,5-diphenyloxazole and 0·02% w/v 1,4-*bis*-[2-(4-methyl-5-phenyloxazolyl)]-benzene was added to each vial which was counted three times in a liquid scintillation counter (Nuclear-Chicago, Mark 1). Standards were used to measure machine efficiency, background count and specific activity of the injected drug. Quenching

was corrected by the external standard ratios method. Calculations were performed on an IBM-360 computer. The radioactivity was expressed as unchanged BHC. The counting efficiency was >80% for bile, urine and faecal homogenate and >70% for blood. The total counts were >25 times background in all samples. The results were expressed as means with standard errors and the significance was determined by Student's *t* test.

Paper chromatography of bile

The 6 h pooled bile of individual rats was analysed for the ^{14}C excreted as unchanged BHC and its metabolites. The bile was extracted according to the method of Nagashima, Levy & Nelson (1968). A 0·5 ml aliquot was mixed with 0·2 ml of citrate-phosphate buffer (0·5 M citric acid/1·0 M disodium hydrogen phosphate, pH 3·0), and extracted 3 times with 3·5 ml aliquots of ethyl acetate. The pooled supernatants were dried at 50° C under a current of nitrogen. Over 90% of the radioactivity was extracted by this method. The residue was redissolved in 100 μl of ethyl acetate and 25 μl applied as a band to a strip of Whatman No. 1 chromatography paper. Aliquots (10 μl) from the ^{14}C-BHC drug solution were run for comparison. The solvent system consisted of equal parts of n-butanol and 3 M aqueous ammonia as used by Christensen (1964). Spots were located by radiochromatographic scanning and counted in a liquid scintillation counter. The radioactivity associated with different R_F-values was expressed as a percentage of the total radioactivity in the bile.

To determine if BHC and its metabolites were present in the bile as conjugates, 0·5 ml aliquots of the pooled bile from each rat were incubated with 0·05 ml of Glusulase (Endo Laboratories, Inc., Garden City, N.Y.) overnight at 37° C, with 0·5 ml acetate buffer (0·2 N acetic acid/0·2 N sodium acetate, pH 5·0). The Glusulase-treated bile was processed as described above.

Results

Excretion of radioactivity into the bile

Radioactivity in bile samples taken after i.v. injection of ^{14}C-BHC or ^{14}C-BHC and salicylate reached a peak in 45–60 min and gradually declined thereafter. The ^{14}C in the bile of salicylate-treated rats was 2-fold higher ($P<0·01$ to 0·001) than in the BHC-treated controls throughout the 6 h collection period (Fig. 1A). The cumulative amount of radioactivity (expressed as per cent of dose administered)

TABLE 1. *Concentration gradients of ^{14}C-radioactivity from blood to liver, and from blood to bile 6 h after i.v. injection of either ^{14}C-bishydroxycoumarin (^{14}C-BHC, 15 mg/kg), or ^{14}C-BHC (15 mg/kg) and sodium salicylate (SAL, 88·9 mg/kg)*

	Treatment	
	^{14}C-BHC	^{14}C-BHC+SAL
Blood conc. (μg/ml)	54·90±2·0	27·00±1·0*
Liver conc. (μg/g)	55·00±2·5	81·10±1·4*
Bile conc. (μg/ml)	124·00±9·4	226·10±8·7*
Liver/blood ratio	1·01±0·08	3·02±0·10
Bile/blood ratio	2·13±0·14	8·23±0·45

Values, expressed as unchanged BHC, represent the mean ± S.E.M. from four rats. * Significantly different at $P<0·001$.

excreted into the bile during this period was about 2·4 times greater in the ^{14}C-BHC + salicylate group than in the ^{14}C-BHC group (Table 2).

The concentration of BHC and its metabolites in the blood, bile and liver of rats 6 h after treatment is shown in Table 1. The data indicate that, irrespective of treatment, the greatest concentration gradient of ^{14}C is from the liver to the bile rather than from the blood to the liver.

Effect of salicylate on bile flow, bile/blood ratio and biliary clearance of ^{14}C-bishydroxycoumarin

Changes in the bile flow rate during the 6 h period following drug injection are illustrated in Figure 2A. Bile flow was increased significantly ($P<0·01$) by salicylate treatment. The maximal choleretic effect ($3·4 \pm 0·3$ (ml/h)/kg) was reached 4 h after salicylate administration.

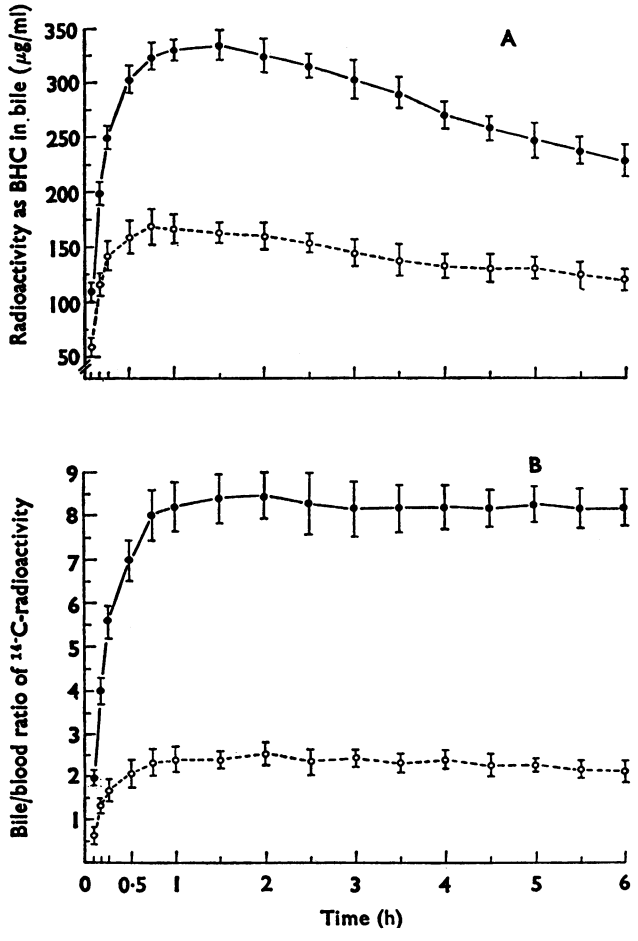

FIG. 1. Bile concentration (A) and bile/blood ratio (B) of ^{14}C-radioactivity expressed as unchanged ^{14}C-bishydroxycoumarin (^{14}C-BHC) during the 6 h period following intravenous administration of ^{14}C-BHC (15 mg/kg, ○– –○) alone and with sodium salicylate (88·9 mg/kg, ●——●) to groups of 5 and 4 rats, respectively. The vertical bars illustrate the range of standard error ($P<0·01$ to 0·001).

The bile/blood ratio of ^{14}C started rising 5 min after each treatment, reaching a plateau in 45–60 min (Fig. 1B). This ratio was 2:1 in the BHC-treated group and 8:1 in the BHC+salicylate-treated rats during most of the 6 h observation period.

The biliary clearance of radioactivity (Fig. 2B), calculated as the product of bile flow rate ((ml/h)/kg) and the bile/blood ratio, was small and constant following

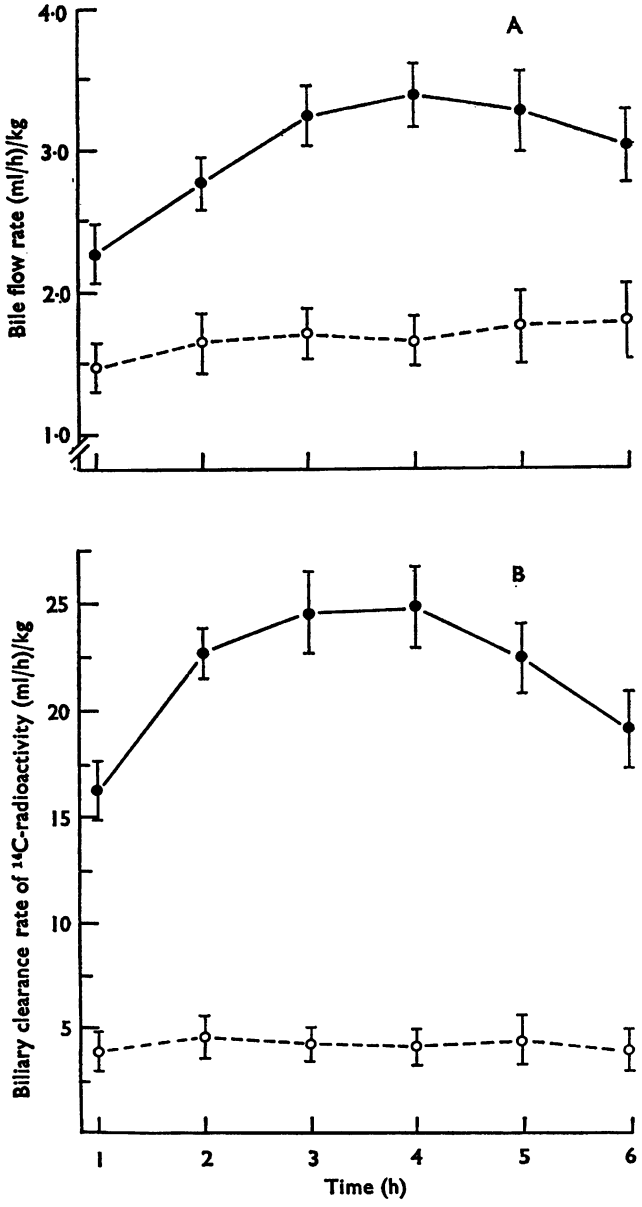

FIG. 2. The bile flow rate (A) and biliary clearance of ^{14}C-radioactivity (B) during the 6 h period following intravenous injection of ^{14}C-bishydroxycoumarin (15 mg/kg, ○– – –○) alone and with sodium salicylate (88·9 mg/kg, ●——●) to groups of 5 and 4 rats, respectively. The vertical bars indicate the range of standard error ($P<0{\cdot}01$ to 0·001).

intravenous administration of BHC alone. When salicylate was administered with the anticoagulant the clearance started rising during the first hour and reached its highest value (24·7 ± 2·1 (ml/h)/kg) 3 to 4 h after drug injection. Biliary clearance in the salicylate-injected rats was 4–5 times greater ($P<0.001$) at all times than in the BHC-injected animals. This was due to two factors: (1) the enhanced bile flow and, (2) the high bile/blood ratio resulting from the increased biliary excretion of radioactivity under the influence of salicylate.

Enterohepatic circulation of ^{14}C-bishydroxycoumarin

The total amount of ^{14}C contained in blood samples taken at timed intervals from sham-operated and bile duct cannulated rats, during the 6 h following the intravenous injection of ^{14}C-BHC alone and with salicylate is shown in Figs. 3A and 3B.

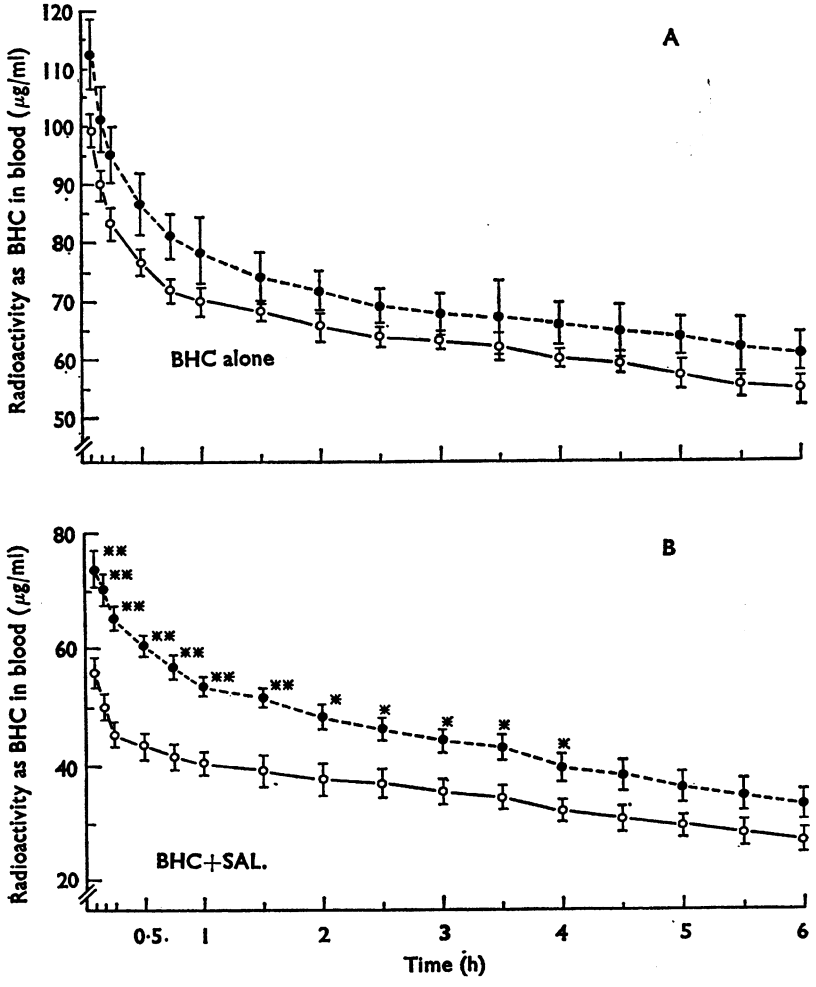

FIG. 3. Concentration of ^{14}C-radioactivity (expressed as unmetabolized bishydroxycoumarin, BHC) in blood samples taken from sham-operated (●– – –●), and bile duct cannulated (○——○) rats during the 6 h period following intravenous injection of either ^{14}C-BHC (15 mg/kg) alone (A) or ^{14}C-BHC (15 mg/kg) with sodium salicylate (SAL, 88·9 mg/kg; B). Each point indicates the mean of values from 4 to 5 rats and the bars illustrate the range of standard error. $^{**}P<0.005$; $^{*}P<0.05$.

respectively. The radioactivity was higher in the blood of sham-operated rats throughout the whole period although the differences were significant only up to the fourth hour in the group administered both drugs.

The total amount of ^{14}C (expressed as per cent of dose of BHC administered) excreted into the bile, faeces and urine of rats where bile was collected (group 1), or allowed to enter the intestine (sham-operated, group II), is shown in Table 2. The total activity recovered in bile, faeces and urine in group I was $14{\cdot}6 \pm 2{\cdot}8\%$ of the injected dose, the greater part being found in the bile ($12{\cdot}3 \pm 2{\cdot}7\%$). The total excretion of activity in group II was $8{\cdot}2 \pm 1{\cdot}1\%$ of the injected dose, the largest amount being present in the faeces ($6{\cdot}6 \pm 0{\cdot}7\%$). These results suggest that when BHC was given by itself about 6% of the dose underwent enterohepatic circulation. Following the administration of ^{14}C-BHC and salicylate $33{\cdot}4 \pm 3{\cdot}9\%$ of the dose was excreted in the bile duct-cannulated rats, the largest portion again appearing in the bile ($29{\cdot}3 \pm 2{\cdot}5\%$). These data indicate that about 11% of the dose of radioactivity excreted into the bile underwent biliary recycling. This would account for the higher concentrations of ^{14}C in the blood of the sham-operated salicylate-treated rats (Fig. 3B).

TABLE 2. *Excretion of ^{14}C-radioactivity into the bile, faeces and urine of rats during 6 h following the intravenous administration of ^{14}C-bishydroxycoumarin (^{14}C-BHC, 15 mg/kg), or ^{14}C-BHC (15 mg/kg) and sodium salicylate (SAL, 88·9 mg/kg)*

Treatment	Group	Activity recovered (% of dose)			
		Bile	Faeces	Urine	Total
^{14}C-BHC alone	I	$12{\cdot}3 \pm 2{\cdot}7$	$1{\cdot}1 \pm 0{\cdot}1$	$1{\cdot}2 \pm 0{\cdot}3$	$14{\cdot}6 \pm 2{\cdot}8$*
	II	—	$6{\cdot}6 \pm 0{\cdot}7$	$1{\cdot}7 \pm 0{\cdot}4$	$8{\cdot}2 \pm 1{\cdot}1$
^{14}C-BHC+SAL	I	$29{\cdot}3 \pm 2{\cdot}5$	$1{\cdot}7 \pm 0{\cdot}1$	$2{\cdot}3 \pm 0{\cdot}3$	$33{\cdot}4 \pm 3{\cdot}9$*
	II	—	$17{\cdot}9 \pm 1{\cdot}6$	$4{\cdot}2 \pm 0{\cdot}4$	$22{\cdot}1 \pm 1{\cdot}2$

Group I represents bile duct-cannulated rats and group II sham-operated rats. Values indicate the mean ± S.E.M. from four rats in each group. * Significantly different from group II at $P<0{\cdot}05$.

The largest amount of ^{14}C in urine was recovered from the sham-operated salicylate-injected group (Table 2). Coumarin metabolites are generally less protein-bound and more water soluble than the parent drugs (Koch-Weser & Sellers, 1971a). It is therefore, possible that some of the BHC metabolites are excreted into the urine while undergoing enterohepatic circulation. The source of radioactivity in the faeces of the bile duct-cannulated rats is apparently diffusion of ^{14}C-BHC and its metabolites from the circulation across the intestinal wall.

Biliary excretion of bishydroxycoumarin and metabolites

The amount of BHC and its major metabolites excreted into the bile in 6 h following i.v. injection of ^{14}C-BHC is summarized in Table 3. The results are expressed as a percentage of the total amount of radioactivity applied to the chromatographic paper. In the untreated bile, 45% of the radioactivity was found very close to the origin and 15·3% corresponded to unchanged BHC. Incubation of the bile with Glusulase indicated that about 32% of the radioactivity was associated with conjugates of BHC metabolites and 8% with conjugates of unchanged BHC. Treatment with salicylate did not alter either the proportion or the pattern of the

radioactive metabolites excreted in the 0–6 h pooled bile. The data suggest that salicylate treatment does not induce the formation of additional metabolites of BHC in the rat liver.

TABLE 3. *R_F-values and distribution (%) of ^{14}C-bishydroxycoumarin (^{14}C-BHC) metabolites occurring in the 6 h pooled biles from four different rats following injection of ^{14}C-BHC (i.v., 15 mg/kg)*

^{14}C-metabolite No.	R_F-value		Total radioactivity in the bile (%)	
	Untreated bile	Glusulase-treated bile	Untreated bile	Glusulase-treated bile
1	0·07 (0·06–0·08)	0·09 (0·09–0·10)	45·0 (42·0–48·0)	11·6 (8·9–13·7)
2	0·21 (0·18–0·25)	0·22 (0·20–0·24)	15·4 (12·2–19·4)	9·3 (7·5–11·6)
3	0·47 (0·45–0·50)	0·50 (0·48–0·52)	14·4 (12·7–17·6)	19·3 (13·4–25·1)
4	0·69 (0·64–0·76)	0·66 (0·65–0·69)	10·0 (8·2–12·7)	36·0 (28·4–40·2)
Unchanged ^{14}C-BHC	0·83 (0·80–0·85)	0·80 (0·77–0·83)	15·3 (12·5–18·4)	23·9 (20·8–28·7)

The glucuronides of ^{14}C-BHC metabolites and the parent drug were hydrolyzed with Glusulase. Values represent the means and figures in parentheses indicate the ranges.

Discussion

A wide variety of drugs have been alleged to interact with the coumarin anticoagulants both in man and experimental animals (O'Reilly & Aggeler, 1970; Koch-Weser & Sellers, 1971a, b). Drugs can modify the anticoagulant action of coumarins by a variety of mechanisms, for example, by altering the amount and rate of absorption from the gastrointestinal tract, by interfering with protein binding and by affecting the metabolic transformation and the ultimate elimination of the anticoagulant.

BHC is extensively bound to plasma proteins, predominantly to serum albumin (Garten & Wosilait, 1971), and the high degree of binding affects its pharmacological and pharmacokinetic properties. Many drugs can displace coumarins from albumin binding sites and thereby can alter their hypoprothrombinemic response. Thomas *et al.* (1973) found that the administration of salicylate (88·9 mg/kg) with BHC (15 mg/kg) reduced the half-life of the latter in rats to 2·9 h compared with 7·7 h in the BHC-treated controls and further, that salicylate displaced BHC from its binding sites on plasma proteins. Since BHC is metabolized by the microsomal enzymes in the liver, the displacement by salicylate might be expected to increase the availability of free BHC to the hepatic cells, thereby enhancing its metabolism and the excretion of both metabolic products and unchanged drug into the bile. As a consequence the concentration of radioactivity in the blood would be reduced, as was observed in the present investigation.

The increased liver concentration of BHC in the salicylate-treated rats observed in this study and by Thomas *et al.* (1973) does not correlate with the pharmacological antagonism observed after 18 h by Zawidzka *et al.* (1972). However, it is likely that increased elimination into the bile will eventually result in lower liver levels of BHC. Another possibility is that salicylate partially antagonizes the action of BHC in rat liver. This would occur if salicylate has a high affinity for, but low intrinsic activity at, the site of action.

It has been postulated that active secretory processes are involved in the transfer of compounds from blood to bile for which the bile/blood ratio is greater than unity (Smith, 1966). In the present experiments the concentration of radioactivity

in the bile from BHC-treated rats was twice that in the blood, and when salicylate was present the concentration of radioactivity was over 8-fold higher in the bile than in the blood (Table 1). In view of these observations, possibly BHC-metabolites, as well as unchanged BHC, are actively transferred across the hepatic membrane into the bile. It would appear that this process may be stimulated by salicylate.

Several substances are known to affect the composition and flow rate of bile in the rat (Slater & Delaney, 1971 ; Koss, Pelzer & Kopitar, 1971). The choleretic effect of salicylate observed in the present experiments is in agreement with earlier observations (Okada, 1915 ; Schmidt *et al.*, 1938). The negligible effect of BHC on the bile flow rate confirms the observations of Husain, Wosilait & Eisenbrandt (1971).

Several investigators have noted that the urinary excretion of BHC is low. The present study confirms that the kidney is a minor route of excretion, since even in the salicylate-treated rats only 4·2% of the dose was recovered in the urine.

Burns, Weiner, Simson & Brodie (1953) reported that following intravenous administration of ethylbiscoumacetate in man, up to 15% of the dose appears in the bile and is reabsorbed from the intestine, metabolized and excreted into the urine. The results of the present study suggest that about 11% of the ^{14}C excreted into the rat bile is reabsorbed into the circulation within 6 h following combined treatment with ^{14}C-BHC and salicylate compared to 6% reabsorption after injection of ^{14}C-BHC alone. The ^{14}C reabsorbed from the intestine was responsible for the relatively high blood levels of activity observed in the sham-operated rats. About 15% of the bile ^{14}C constituted unmetabolized BHC. The coumarin anticoagulants are lipid soluble and able to gain access to cells with relative ease compared with their water soluble polar metabolites (Koch-Weser & Sellers, 1971a). Therefore, it is likely that the major part of the radioactivity absorbed from the intestine was contributed by the unmetabolized drug. However, the present study does not rule out the possible reabsorption of metabolites of BHC. Biliary recycling of unchanged BHC might contribute to the prolonged anticoagulant action characteristic of this coumarin. Metabolites of BHC have been recovered from rat faeces (Christensen, 1965), and from human urine (Weiner, Shapiro, Axelrod, Cooper & Brodie, 1950), but their exact structure is unknown.

In conclusion, the present experiments demonstrate that the co-administration of salicylate and BHC to the rat accelerates the biliary excretion and elimination from the body of the anticoagulant and its metabolites. This may explain, in part at least, the antagonistic action of salicylate on the pharmacological effect of BHC in this species.

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