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THE EFFECT OF MALE FERN EXTRACT ON BILIARY SECRETION

BY

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Extract of male fern (*Dryopteris felix-mas*) has been widely used in the treatment of tapeworm infestation. The extract produces toxic effects, including gastrointestinal symptoms, impairment of vision and jaundice. Nosslin (1963) showed that male fern extract produced an unconjugated hyperbilirubinaemia together with bromsulphthalein retention in patients treated for tapeworm infestation; he suggested that male fern extract reduced the uptake of bilirubin from the blood by liver cells.

This paper describes a study of the effect of male fern extract on glucuronide conjugation in rat and rabbit liver *in vitro* and on the excretion of pigments by rat liver into the bile *in vivo*, with a view to determining the site of action of male fern extract which causes jaundice.

METHODS

Materials

The British Pharmacopoeial Extract of Male Fern was used.

Acceptor substances. *o*-Aminophenol (Koch-Light) was resublimed before use, and ascorbic acid (analytical grade) was added to the *o*-aminophenol solution to minimize oxidation. Bilirubin (B.D.H.), 10 mg, was dissolved in 0.15 ml. 0.25-N sodium hydroxide with stirring. Phosphate-bicarbonate solution, 0.85 ml., was added and the solution was centrifuged to remove undissolved material; this was used in the *in vitro* experiments.

Uridine diphosphate glucuronic acid "uridine diphospho-glucuronic acid," 98 to 100% purity, ammonium salt (Sigma Chemical Co.).

Bilirubin for infusion was prepared by dissolving 25 mg of bilirubin in 10 ml. of an isotonic solution containing 0.5 g of sodium carbonate and 0.52 g of sodium chloride per 100 ml. Bromsulphthalein (Samoore) was infused in a concentration 20 mg/ml. Indocyanine green (Batch No. 5564, Koch-Light) was infused in a concentration 1 mg/ml.

Conjugated bilirubin was added in the *in vitro* experiments as undiluted human bile obtained from patients in whom a T-tube had been inserted into the common bile duct. For the *in vivo* experiments conjugated bilirubin was obtained by infusing a rat with bilirubin and collecting the bile; this was then diluted with 0.85% (w/v) saline. The concentration of conjugated bilirubin was determined by the method of Malloy & Evelyn (1937).

Phosphate-bicarbonate solution, *p*H 7.4, contained (mM) 27 $NaHCO_3$, 123 NaCl, 5 KCl, 1.2 KH_2PO_4 and 1.2 $MgCl_2$. The solution was gassed with 5% carbon dioxide and 95% oxygen for 10 min.

Rate of conjugation

In glucuronide synthesis a glucuronyl group is transferred from uridine diphosphate glucuronate under the influence of the microsomal enzyme uridine diphosphate transglucuronylase (uridine diphosphate

glucuronate glucuronyl transferase, acceptor unspecific, EC 2.4.1.17) to an acceptor which may be one of a wide variety of compounds (for example, *o*-aminophenol and bilirubin). The rate of conjugation of bilirubin and *o*-aminophenol was estimated by determining glucuronide synthesis *in vitro*. Uridine diphosphate glucuronic acid was added in the homogenate experiments.

Bilirubin as an acceptor substrate. Synthesis of bilirubin glucuronide in sliced rat liver tissue was determined by the method of Lathe & Walker (1958). Synthesis in rabbit liver homogenates was by a slight modification of the method of Lathe & Walker (1958) with added uridine diphosphate glucuronic acid in a final concentration of 0.3 mM. Bilirubin in tissues was estimated by the method described by Hargreaves (1965).

o-Aminophenol as acceptor substrate. Synthesis of *o*-aminophenyl glucuronide in rat liver slices was determined by the method of Levvy & Storey (1949). Synthesis in rabbit liver homogenates was by the method of Stevenson & Dutton (1962).

Uptake of conjugated bilirubin

The rate of uptake of conjugated bilirubin by rat liver slices was determined by shaking liver slices of approximately 200 mg in conical flasks containing 3 ml. of phosphate-bicarbonate solution and 0.15 mg of conjugated bilirubin for 1 hr at 37° C with air as the gas phase. Normal liver slices and slices denatured by heating at 80° C for 5 min were used. Conjugated bilirubin was determined in the slices as described by Hargreaves (1965).

Animal experiments

The effect of male fern extract on the biliary excretion of bilirubin, bromsulphthalein and indocyanine green was determined in rats. Male fern extract (0.02 ml./100 g body weight) was injected intraperitoneally 1 hr before the infusion of bilirubin or the dyes. Male white rats (weight 260 to 280 g) were anaesthetized with 0.07 ml./100 g of pentobarbitone sodium solution (32 mg/ml.) and the bile duct was cannulated (Weinbren & Billing, 1956). Bilirubin and the dyes were infused intravenously and the biliary excretion of each was determined. This method enabled a study of male fern extract on biliary excretion to be made.

Excretion of injected bilirubin (unconjugated). Bilirubin, 18 mg in bicarbonate-saline solution, was given intravenously over a period of 30 min. Bile was collected for three 15-min periods and the bilirubin (total and conjugated) was estimated by the method of Malloy & Evelyn (1937). The maximum rate of bilirubin excretion was determined over the 15- to 30-min period and was expressed as μg of bilirubin excreted per 100 g body weight per min. After 45 min the rat was killed and the total and conjugated bilirubin contents of the liver were determined.

Excretion of injected bilirubin (conjugated). Rat bile containing 20 mg of conjugated bilirubin was diluted with 0.85% (w/v) saline and given intravenously to rats over a period of 30 min. Bile was collected for three 15-min periods and the conjugated bilirubin was estimated by the method of Malloy & Evelyn (1937). The maximum rate of conjugated bilirubin excretion for the 15- to 30-min period was determined and expressed as μg of conjugated bilirubin excreted per 100 g body weight per min.

Excretion of bromsulphthalein. Bromsulphthalein (20 mg/100 g body weight) was infused intravenously over a period of 30 min, 15-min collections of bile were made and the bromsulphthalein estimated (Varley, 1962). The results were expressed as μg of bromsulphthalein excreted per 100 g body weight per min for the period 15 to 30 min.

Excretion of indocyanine green. Indocyanine green (1 mg/100 g body weight) was dissolved in water (1 mg/ml.) and infused intravenously for 30 min into anaesthetized rats. Collections of bile were made over 15-min periods and the indocyanine green was estimated by dilution and measurement of the extinction at 805 mμ. The results were expressed as μg of indocyanine green excreted per 100 g body weight per min for the period 15 to 30 min.

RESULTS

In vitro *experiments*

The addition of male fern extract lowered the rates of conjugation of bilirubin and *o*-aminophenol by rat liver slices and decreased the concentration of both conjugates in

TABLE 1

EFFECT OF MALE FERN EXTRACT ON BILIRUBIN CONJUGATION AND BILIRUBIN CONTENT IN RAT LIVER SLICES

Values are means of four experiments

Dose of male fern extract (ml.)	Bilirubin: Rate of conjugation (mμmoles/mg/hr)	Bilirubin: In slice: Total (μg/g)	Bilirubin: In slice: Conjugated (%)	o-Aminophenol Rate of conjugation (mμmoles/mg/hr)
0	0·09	82	67	0·9
0·001	0·07	63	72	0·68
0·002	0·032	68	57	0·29
0·003	0·023	81	44	0·18
0·005	0·016	89	27	0·08

TABLE 2

EFFECT OF MALE FERN EXTRACT ON CONJUGATION IN RABBIT LIVER HOMOGENATES

Values are means of four experiments with ranges in parentheses

Dose of male fern extract (ml.)	Rate of conjugation (mμmoles/mg/hr): o-Aminophenol	Rate of conjugation (mμmoles/mg/hr): Bilirubin
0	5·3 (4·3–7·1)	0·44 (0·4–0·47)
0·001	0·36 (0·17–0·5)	0·17 (0·12–0·22)
0·002	0·12 (0·07–0·15)	0·02 (0–0·04)
0·003	0·24 (0·15–0·32)	0
0·005	0·17 (0·02–0·3)	0
0·01	0	0

TABLE 3

EFFECT OF MALE FERN EXTRACT ON ACCUMULATION OF CONJUGATED BILIRUBIN IN RAT LIVER SLICES

Values are means of four experiments with ranges in parentheses

Dose of male fern extract (ml.)	Conjugated bilirubin (μg/g) in slice: Normal	Conjugated bilirubin (μg/g) in slice: Denatured
0	117 (86–141)	440 (401–498)
0·01	79 (55–97)	286 (212–393)
0·02	53 (25–75)	200 (126–320)
0·03	35 (16–67)	225 (165–335)
0·05	38 (14–63)	200 (133–301)

TABLE 4

EFFECT OF MALE FERN EXTRACT ON MAXIMUM HEPATIC CLEARANCE IN WISTAR RATS

Values are means

Substance excreted	No. of expts.	Male fern extract (ml./100 g)	Maximum hepatic clearance (μg/100 g/min)	P
Bilirubin	14	0	74	
	6	0·02	47	<0·02
	4	0·04	27	<0·001
Conjugated bilirubin	6	0	43	
	6	0·02	26	<0·001
Bromosulphthalein	7	0	73	
	7	0·02	47	<0·02
Indocyanine green	12	0	4·8	
	5	0·02	5·2	

TABLE 5

EFFECT OF MALE FERN EXTRACT ON LIVER AND SERUM PIGMENTS

Values are means. Bilirubin given, 7·8 mg/100 g body weight; conjugated bilirubin, 8·0 mg/100 g body weight

Pigment	No. of expts.	Male fern extract (ml./100 g)	Liver bile pigments		Serum bile pigments	
			Total (mg/g)	Conjugated (%)	Total (mg/100 ml.)	Conjugated (%)
Bilirubin	14	0	1·01	75	38	17·0
	6	0·02	1·12	49	39·5	17·3
	4	0·04	1·64	36	45	18
Conjugated bilirubin	6	0	0·3	77	21·5	90
	6	0·02	0·35	77	20·8	90

the fluid medium. It also reduced the amount of conjugated bilirubin formed in the slices (Table 1).

Conjugation by uridine diphosphate transglucuronylase was examined in rabbit liver homogenate incubated in a medium containing excess uridine diphosphate glucuronic acid. Male fern extract inhibited *o*-aminophenol conjugation and bilirubin conjugation in rabbit liver homogenates (Table 2).

The effect of male fern extract on conjugated bilirubin uptake was studied *in vitro* (Table 3). The extract reduced uptake in living and denatured slices which suggests that this was a physical phenomena. The amount of extract required to reduce uptake of conjugated bilirubin was ten times that required to inhibit conjugation.

In vivo *experiments*

The maximum hepatic clearance of bilirubin, conjugated bilirubin bromsulphthalein and indocyanine green is shown in Table 4 in normal rats and in rats treated with extract of male fern. Male fern extract reduced the maximum hepatic clearance of bilirubin, conjugated bilirubin and bromsulphthalein but not the clearance of indocyanine green. Doubling the dose of male fern extract reduced the maximum hepatic clearance of bilirubin further.

The effect on conjugation was studied further by determining the concentration of " conjugated " pigment in the liver after bilirubin infusion. The percentage of " conjugated " bilirubin in the animals treated with male fern extract was significantly decreased ($P<0.01$) compared with that for the control animals (Table 5). The total bilirubin levels showed that in the intact rat, at this dose level, entry of bilirubin into the liver was not impaired.

DISCUSSION

Male fern extract is an ether extract of *Dryopteris felix-mas* rhizomes containing a number of substances including filicin, flavaspidic acid and aspidinol (Blakemore, Bowden, Broadbent & Drysdale, 1964). Runeberg (1962) showed that flavaspidic acid and aspidinol uncoupled oxidative phosphorylation.

Male fern extract produces an unconjugated hyperbilirubinaemia in man, and this could be due to defective uptake or conjugation of bilirubin by the liver cell. Nosslin (1963) suggested that the extract caused jaundice by inhibiting uptake of bilirubin by liver cells.

The addition of male fern extract to rat liver slices and rabbit liver homogenates reduced the rates of conjugation of *o*-aminophenol and bilirubin by these tissues. The rate of conjugation of the acceptor substances could be reduced in rat liver slices by male fern extract preventing the uptake of unconjugated bilirubin by rat liver slices. Table 1 shows that the entry of unconjugated bilirubin into the liver slices is not impaired but the amount of conjugated bilirubin in the slice is decreased. This suggested that the enzyme uridine diphosphate transglucuronylase was inhibited in rat liver slices, thereby decreasing the amount of conjugated bilirubin formed and released by the rat liver slices.

The enzyme uridine diphosphate transglucuronylase is an unstable microsomal enzyme; the enzyme activity of tissue homogenates and microsomal preparations declines rapidly on ageing (Dutton & Storey, 1954). The effect of inhibitors on the enzyme has been studied using fresh liver homogenate preparations (Hargreaves & Lathe, 1963; Storey, 1965); addition of male fern extract to rabbit liver homogenates reduced the rates of conjugation of *o*-aminophenol and bilirubin.

In animal experiments male fern extract decreased the maximum hepatic clearance of bilirubin, and this could be due to decreased uptake of bilirubin, decreased uridine diphosphate transglucuronylase activity or impaired excretion of the conjugated pigment. The serum bilirubin levels were not significantly different in the treated and control animals nor was the amount of unconjugated bilirubin in the livers of treated animals decreased. This suggested that entry of unconjugated bilirubin was not impaired. The amount of " conjugated " bilirubin in the livers of treated animals was decreased, and the amount was less with increasing amounts of administered male fern extract.

Male fern extract decreased the maximum hepatic clearance of conjugated bilirubin; it is unlikely that uptake into the liver was reduced because there was no significant difference between the serum levels and the liver content of conjugated bilirubin in treated and untreated animals. In the *in vitro* experiments small amounts of male fern extract did not decrease the uptake of conjugated bilirubin by rat liver slices.

Male fern extract inhibited conjugation *in vitro* and *in vivo*. It also reduced the biliary excretion of conjugated bilirubin. This was investigated further by studying the effect of male fern extract on the biliary excretion of bromsulphthalein and indocyanine green. Bromsulphthalein, the disulphonate derivative of phenoltetrabromphthalein, is excreted in rat bile partly in an unconjugated form and partly conjugated with glutathione. The enzyme is in the soluble fraction of rat liver (Combes & Stakelum, 1960). Javitt (1965) has shown that the monosulphonate derivative of phenoltetrabromphthalein can be excreted as a glutathione-glucuronide conjugate. Indocyanine green is excreted as the free dye (Caesar, Shaldon, Chiandussi, Guevara & Sherlock, 1961). Male fern extract decreased the excretion of bromsulphthalein but not that of indocyanine green. It is possible that bilirubin, conjugated bilirubin and bromsulphthalein are excreted by rat liver into the bile by a system closely linked with uridine diphosphate transglucuronylase. Bilirubin requires the latter for excretion and it is conceivable that bromsulphthalein may also require this enzyme because a derivative (the monosulphonate) is excreted as a glucuronide. Indocyanine green is excreted as the free dye and may therefore be excreted by a different mechanism.

It does not necessarily follow that a substance preventing the excretion of conjugated bilirubin will cause a conjugated hyperbilirubinaemia. Novobiocin, which causes an

unconjugated hyperbilirubinaemia in man (Sutherland & Keller, 1961), reduced bilirubin, conjugated bilirubin and indocyanine green clearance in rats and inhibited conjugation *in vitro* in the rat and rabbit (Hargreaves & Lathe, 1963). Buniodyl produced an unconjugated hyperbilirubinaemia in man (Bolt, Dillon & Pollard, 1961), it inhibited conjugation *in vitro* and reduced the biliary excretion of bilirubin, conjugated bilirubin and indocyanine green (Hargreaves & Lathe, 1963; Billing, Maggiore & Cartter, 1963). The effect of male fern extract on conjugation and biliary secretion is unlike that of buniodyl because buniodyl does not reduce the conjugated liver pigments after bilirubin infusion (Billing *et al.*, 1963) as does male fern extract.

Male fern extract produces an unconjugated hyperbilirubinaemia in man, and it has been suggested that this is due to reduced uptake of bilirubin by the liver. The evidence presented here shows that male fern extract inhibits uridine diphosphate transglucuronylase and reduces the biliary excretion of bilirubin, conjugated bilirubin and bromsulphthalein. This may be because the processes of conjugation by uridine diphosphate transglucuronylase and biliary excretion of these substances are closely linked in the liver cell.

SUMMARY

1. Male fern extract inhibited *o*-aminophenol and bilirubin conjugation *in vitro*.

2. The extract decreased the maximum hepatic clearance of bilirubin, conjugated bilirubin and bromsulphthalein, but not that of indocyanine green.

3. Male fern extract decreased the conjugated bilirubin content of rat liver in slices conjugating bilirubin and of rat liver maximally secreting bilirubin.

4. The unconjugated hyperbilirubinaemia produced by male fern extract is probably due to inhibition of uridine diphosphate transglucuronylase.

5. It is suggested that uridine diphosphate transglucuronylase is closely linked with the excretion of bilirubin and bromsulphthalein but not so closely linked with indocyanine green excretion.

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