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THE EFFECT OF DIETHYAMINOETHYL DIPHENYLPROPYLACETIC ACID (SKF 525-A) ON URIDINE 5′-PYROPHOSPHATE GLUCURONYLTRANSFERASE

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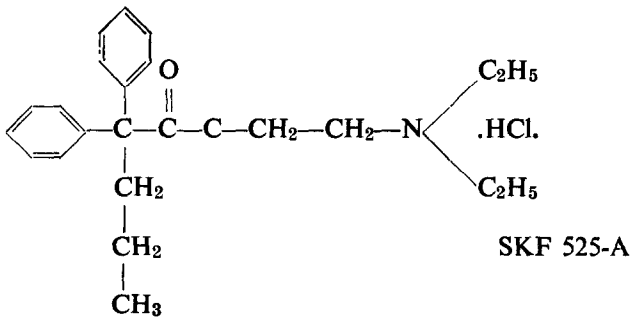
Abstract—The effect of SKF 525-A on UDP glucuronyltransferase has been investigated using *o*-aminophenol, bilirubin and *p*-nitrophenol as substrates. It was found that SKF 525-A inhibited the conjugation of *o*-aminophenol in rat liver slices and homogenates and guinea pig liver homogenates, and *p*-nitrophenol conjugation in guinea pig and rat liver homogenates. Bilirubin conjugation was inhibited in rat liver slices and homogenates, and to a lesser extent in guinea pig liver. Low concentrations of SKF 525-A stimulated *o*-aminophenol conjugation in jaundiced Gunn rat liver homoenates but it was inhibited at higher concentrations. It appears that these results support the concept that there are a number of UDP glucuronyltransferase. The biliary excretion of bilirubin, conjugated bilirubin, bromsulphthalein and indocyanine green was inhibited in rats suggesting that the processes of conjugation and excretion from the liver cells are closely linked.

DIETHYLAMINOETHYL diphenylpropylacetic acid (SKF 525-A) is the best known and most widely used inhibitor of the liver microsomal enzymes which catalyse drug metabolism.



SKF 525-A

The first observations with this compound were made by Cook, Toner and Fellows[1] and Axelrod, Reichenthal and Brodie.[2] The substance increased the duration of the hexobarbitone sleeping time and it has since been shown that the drug inhibits many reactions catalysed by microsomal enzymes. In this paper the effect of SKF 525-A on the microsomal enzyme UDP glucuronyltransferase *in vitro* has been investigated using *o*-aminophenol, bilirubin and *p*-nitrophenol as substrates. Because of the possible link between conjugation and excretion by the liver its effect on the biliary excretion

of bilirubin, conjugated bilirubin, bromsulpthalein and indocyanine green *in vivo* has also been investigated.

MATERIALS AND METHODS

Materials

Acceptor substances: (1) *o*-aminophenol (Koch-Light Ltd) was resublimed before use and ascorbic acid (AR) was added to the *o*-aminophenol solution to minimise oxidation. (2) 10 mg bilirubin (British Drug Houses, Ltd.) was dissolved in 0·15 ml 0·25 N-NaOH with stirring and 0·85 ml phosphate-bicarbonate solution was added. This was then mixed and centrifuged to remove any material not in solution. This bilirubin solution was used in the *in vitro* experiments.

UDP glucuronic acid: the ammonium salt of uridine diphosphoglucuronic acid (98–100 per cent quoted purity) from Sigma Chemical Co., St. Louis, Mo., U.S.A. was used.

Bilirubin for infusion was prepared by dissolving 25 mg bilirubin in 10 ml of an isotonic solution containing 0·52 g Na_2CO_3 and 0·5 g NaCl per 100 ml. Conjugated bilirubin was obtained for the *in vivo* experiments by infusing a rat with bilirubin and collecting the bile. This was then diluted with 0·85% sodium chloride solution and the concentration of conjugated bilirubin was determined by the method of Malloy and Evelyn.[3] Bromsulphthalein (Samoore Ltd.) was infused in a concentration of 5 mg/ml. Indocyanine green (Batch No. 17443 Koch-Light Ltd.) was infused in a concentration 1 mg/ml. All other chemicals were from British Drug Houses Ltd.

Phosphate-bicarbonate solution (pH 7·4).[4] This was 27 mM-$NaHCO_3$, 123 mM-NaCl, 5 mM-KCl, 1·2 mM-KH_2PO_4 and 1·2 mM-$MgCl_2$. The solution was gassed with O_2 and CO_2 (95:5) for 10 min.

Animals

Male Wistar rats and male guinea pigs were bred from stock obtained commercially. Jaundiced Gunn[5] rats were bred from stock kindly given by Dr. B. H. Billing. All animals were fed immediately before the experiment. For the *in vitro* experiments each animal was killed by stunning and cervical dislocation and the liver removed and placed immediately in ice–cold phosphate-bicarbonate solution. Liver slices, 0·1 mm thick, were cut by hand with a razor blade. Homogenates were prepared by grinding 1 g liver in a teflon-glass homogeniser with 9 ml 0·154 M-potassium chloride solution containing $3{\cdot}2 \times 10^{-4}$M potassium bicarbonate solution.

Rate of conjugation

In glucuronide synthesis a glucuronide group is transferred from UDP-glucuronate under the influence of the microsomal enzyme UDP glucuronyltransferase (UDP-glucuronate glucuronyl transferase (acceptor unspecific) EC 2.4.1.17) to an acceptor which may be one of a variety of compounds (e.g. *o*-aminophenol, *p*-nitrophenol and bilirubin). The rate of conjugation of bilirubin, *o*-aminophenol and *p*-nitrophenol was estimated by determining the amount of glucuronide synthesised *in vitro*. UDP-glucuronate was added in the homogenate experiments to ensure that sufficient was present in the medium.

Bilirubin as acceptor substrate. Synthesis of bilirubin glucuronide in sliced rat liver tissue was determined by the method of Lathe and Walker.[4] Synthesis in guinea pig and rat liver homogenates was determined by a slight modification of the method of Lathe and Walker[4] with added UDP-glucuronate in a final concentration 0·3 mM. The amount of bilirubin in tissues was estimated by the method of Hargreaves.[6]

o-*Aminophenol as acceptor substrate.* Synthesis of *o*-aminophenyl glucuronide in rat liver slices was determined by the method of Levvy and Storey.[7] Synthesis in guinea pig and rat liver homogenates was determined by the method of Stevenson and Dutton.[8]

p-*Nitrophenol as acceptor substrate.* Synthesis of *p*-nitrophenyl glucuronide was determined in guinea pig and rat liver homogenates by the method described by Storey[9] based on the method of Isselbacher.[10]

Inhibition experiments. SKF 525-A was added to the reaction mixture to give final concentrations in the range 0·01 mM–10 mM. In the homogenate experiments the incubation was limited to 15 min to preserve linearity of rate with time.

Units. Rates of conjugation are expressed as mμ mole of acceptor conjugated (or glucuronide formed) per mg wet wt. liver per hr at 37°.

Animal experiments

Male Wistar rats were anaesthetised with ether, and the bile duct was cannulated.[11] Aqueous solutions of SKF 525-A in concentrations of 1·5, 3·0 and 4·5 mg/100 g body wt. were given i.v. for 30 min before the subsequent i.v. infusion of the substance under investigation. The biliary excretion of the infused bilirubin or dyes was then estimated and so the effect of SKF 525-A on the biliary excretion of these substances was studied.

Excretion of injected bilirubin (unconjugated). 7 mg bilirubin/100 g body wt. was given i.v. in bicarbonate-saline solution over a period of 30 min. Two 15 min collections of bile were made during this period and the total and conjugated bilirubin estimated in each sample.[3] The rate of bilirubin excretion was maximum during the 15–30 min period and this value was expressed as μg bilirubin excreted/100 g body wt./min.

Excretion of injected bilirubin (conjugated). Rat bile was diluted with 0·85 % sodium chloride solution, and given i.v. in a dose of 7 mg/100 g body wt. to each rat over a 30-min period. Bile was collected during this time in two 15 min samples and the conjugated bilirubin estimated.[3] The maximum rate of conjugated bilirubin excretion was determined as μg conjugated bilirubin excreted/100 g body wt./min for the 15–30 min period.

Excretion of bromsulphthalein. 5 mg/100 g body wt. bromsulphthalein was infused into each rat over a period of 30 min. Two 15 min collections of bile were made for this period and the bromsulphthalein estimated.[12] The results were expressed as μg bromsulphthalein excreted/100 g body wt./min for the 15–30 min period.

Excretion of indocyanine green. An aqueous solution of indocyanine green containing 1 mg/ml was infused i.v. into anaesthetised rats in a dose of 1 mg/100 g body weight for 30 min. Two 15 min collections of bile were made during this period and the indocyanine green estimated by dilution and measurement of the absorbance at 805 mμ. The results were expressed as μg indocyanine green excreted/100 g body wt./min for the 15–30 min period.

RESULTS

In vitro *experiments*

The addition of SKF 525-A lowered the rates of conjugation of *o*-aminophenol by rat liver slices. The rate of bilirubin conjugation was lowered and the amount of conjugated bilirubin in the slice was decreased (Table 1).

Conjugation by UDP glucuronyltransferase was examined in guinea pig, Wistar rat and jaundiced Gunn rat liver homogenates in a medium containing ample UDP-glucuronate and the rates of conjugation of the control samples are shown in Table 2.

TABLE 1. EFFECT OF SKF 525-A ON BILIRUBIN AND *o*-AMINOPHENOL CONJUGATION AND BILIRUBIN CONTENT IN RAT LIVER SLICES

	Bilirubin			*o*-Aminophenol
SKF 525-A conc. (mM)	Rate of conjugation mμ mole/mg/hr	Bilirubin in slice Total μg/g	Conjugated %	Rate of conjugation mμ mole/mg/hr
0	0·097	83	62	0·84
0·01	0·063	80	55	0·59
0·1	0·066	89	47	0·53
1·0	0·034	90	50	0·34
10·0	0	102	17	0·15

Mean of 5 experiments with each substrate.
Bilirubin conjugation: 200 mg rat liver slices were incubated in a 25 ml conical flask containing 0·25 mM bilirubin, serum 14·6% (v/v), the volume being made up to 3 ml with phosphate-bicarbonate solution. *o*-Aminophenol conjugation: 200 mg rat liver slices were incubated in a 25 ml conical flask containing 1·0 mM *o*-aminophenol, 1·0 mM ascorbic acid, serum 14·6% (v/v) the volume being made up to 2·5 ml with phosphate-bicarbonate solution. The flasks were incubated for 2 hr at 37° with air as the gas phase.

TABLE 2. RATES OF GLUCURONIDE SYNTHESIS BY LIVER HOMOGENATES

Acceptor	Species	Acceptor conjugated mμ mole/mg wet wt./hr at 37°
Bilirubin	Guinea pig	0·71 (8)
	Wistar rat	0·34 (7)
	Jaundiced Gunn rat	0 (6)
o-Aminophenol	Guinea pig	8·0 (8)
	Wistar rat	1·7 (8)
	Jaundiced Gunn rat	0·85 (6)
p-Nitrophenol	Guinea pig	4·0 (8)
	Wistar rat	3·7 (8)

Number of experiments in parentheses.
For *o*-aminophenol glucuronide synthesis the tubes contained Tris-HCl buffer pH 7·4 (33 mM), $MgCl_2$ (10 mM), *o*-aminophenol (0·22 mM), ascorbic acid (1·1 mM) UDP glucuronate (0·2 mM) plus water to 0·6 ml. For *p*-nitrophenyl glucuronide synthesis the tubes contained Tris-HCl buffer pH 7·4 (33 mM), *p*-nitrophenol (0·14 mM), UDP-glucuronate (0·1 mM) and water to 0·6 ml. For bilirubin glucoronide synthesis the tubes contained phosphate buffer pH 7·4 (36 mM), $MgCl_2$ (12 mM), bilirubin (0·342 mM), serum 14·6% (v/v), glucuronate (0·3 mM) and water to 0·5 ml 10 mg wet wt. liver homogenate was added to each tube and the tubes were incubated for 15 min with air as the gas phase.

In guinea pig liver homogenates SKF 525-A inhibited conjugation of *o*-aminophenol, *p*-nitrophenol and, to a lesser extent, bilirubin. (Fig. 1). In Wistar rat liver homogenates SKF 525-A lowered the rates of conjugation of all three acceptor substances. (Fig. 2). Jaundiced Gunn rat liver homogenates did not conjugate bilirubin, either in the presence of or absence of SKF 525-A. The rate of conjugation of *o*-aminophenol was

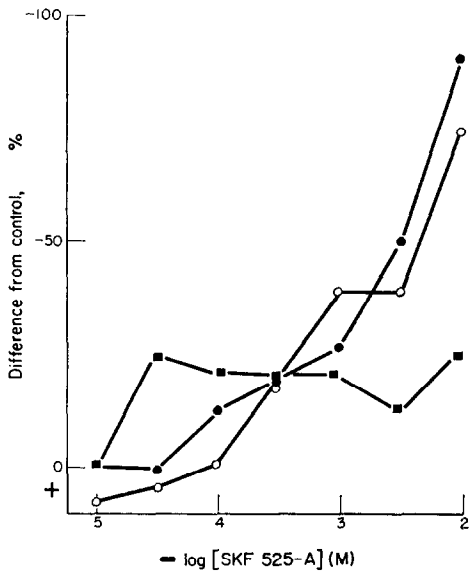


FIG. 1. Effect of SKF 525-A on conjugation in guinea pig liver homogenate. Acceptor substances ●—● *o*-aminophenol. ■—■ bilirubin, ○—○ *p*-nitrophenol. Conditions as in Table 2. Each point is the mean of 6 experiments.

low and in the presence of high concentrations of SKF 525-A the rate was reduced further. Low concentrations of SKF 525-A were found to increase the rate of *o*-aminophenol conjugation by 60 per cent (Fig. 2).

In vivo *experiments*

SKF 525-A decreased the biliary excretion of bilirubin, conjugated bilirubin, bromsulphthalein and indocyanine green when the drug was injected in a dose of 4·5 mg. SKF 525-A/100 g body wt. (Table 3), when smaller doses were injected (1·5 mg and 3·0 mg/100 g body wt.) the biliary excretion of pigment and dyes was not reduced.

DISCUSSION

SKF 525-A prolongs the action of a variety of drugs by inhibiting their metabolism *in vivo*.[13] *In vivo* SKF 525-A must be administered simultaneously with the drug under investigation for that drug action to be prolonged. If, for example, it was administered to animals recovering from sleep after hexobarbitone then SKF 525-A had no effect, whereas chlorpromazine given at that time produced sleep in the animal. Chlorpromazine potentiates the action of barbitone by making the animal more 'sensitive' to the drug.[14] SKF 525-A potentiates hexobarbitone by inhibiting the microsomal enzyme responsible for hexobarbitone metabolism thus preventing its removal. The

mechanism of microsomal enzyme inhibition by SKF 525-A is obscure. It has been shown that the drug does not block NADPH generation or the electron transport mechanism in microsomes:[14] SKF 525-A blocks the metabolism of some lipid soluble substances, for example codeine[15] and hexobarbital;[16] but it does not affect the metabolism of other lipid soluble substances e.g. chlorpromazine.[17]

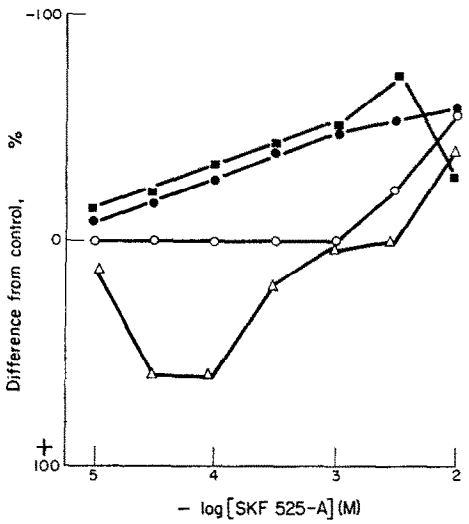


FIG. 2. Effect of SKF 525-A on conjugation in rat liver homogenate. Wistar rat; acceptor substances ●—● *o*-aminophenol, ■—■ bilirubin ○—○ *p*-nitrophenol. Jaundiced Gunn rat; acceptor substance △—△ *o*-aminphenol. Conditions as in Table 2. Each point is the mean of 6 experiments.

TABLE 3. EFFECT OF SKF 525-A ON MAXIMUM HEPATIC CLEARANCE IN WISTAR RATS

	SKF 525-A mg/100 g	No. of expts	Mean maximum hepatic clearance μg/100 g/min	P
Bilirubin	0	14	74	
	1·5	3	80	
	3·0	4	82	
	4·5	5	27	
Conjugated bilirubin	0	6	43	
	4·5	6	26	
Bromsulphthalein	0	7	73	
	1·5	3	76	
	3·0	3	91	
	4·5	6	50	<0·05
Indocyanine green	0	12	4·8	
	3·0	3	4·1	
	4·5	6	3·2	<0·001

Male white rats were anaesthetised with ether and the bile duct cannulated. SKF 525-A was injected i.v. 30 min before infusion of bilirubin (7 mg/100 g) conjugated bilirubin (7 mg/100 g), bromsulphthalein (5 mg/100 g) or indocyanine green (1 mg/100 g) respectively. The biliary excretion of bilirubin,[3] bromsulphthalein[12] and indocyanine green was determined.

SKF 525-A inhibited conjugation of *o*-aminophenol and *p*-nitrophenol and, to a lesser extent, bilirubin in guinea pig liver homogenates. In rat liver homogenates *o*-aminophenol, *p*-nitrophenol and bilirubin conjugation was inhibited as was bilirubin and *o*-aminophenol conjugation in rat liver slices. In the jaundiced Gunn rat, which is deficient in bilirubin UDP glucuronyltransferase, liver homogenates did not conjugate biliburin and conjugation was not stimulated by SKF 525-A. The homogenates, however, conjugated *o*-aminophenol at a low rate. Low concentrations of SKF 525-A stimulated the conjugation of *o*-aminophenol while 10 mM SKF 525-A inhibited conjugation. These results appear to suggest that there may be a number of UDP glucuronyltransferases capable of conjugating different substrates. In guinea pig liver the enzyme conjugating bilirubin is less susceptible to inhibition by SKF 525-A than that conjugating other substrates. It may be different from the enzyme or enzymes conjugating *o*-aminophenol and *p*-nitrophenol. Jaundiced Gunn rat liver contains an enzyme conjugating *o*-aminophenol but it does not conjugate bilirubin. The enzyme conjugating *o*-aminophenol differs from that in the Wistar rat in that it is stimulated by SKF 525-A. This suggests that the enzymes conjugating bilirubin and *o*-aminophenol may be different in jaundiced Gunn rats and that they differ from the enzymes in the Wistar rat. It has been suggested that a number of UDP glucuronyltransferases occur in other species. For example Storey[18] showed that *o*-aminophenol conjugation in mouse liver was more readily inhibited than *p*-nitrophenol conjugation by various agents, and that the two enzyme systems differed in respect of their magnesium requirements. The final proof of the existence of several forms of UDP glucuronyltransferase depends on adequate solubilisation of microsomal UDP glucuronyltransferase and investigation of its kinetics.

SKF 525-A must act at some point in the liver cell whence it can inhibit both bilirubin and *o*-aminophenol conjugation by UDP glucuronyltransferase and also excretion from the liver cell into the bile. The fact that SKF 525-A decreased the biliary excretion of bilirubin, conjugated bilirubin, bromsulphthalein and indocyanine green suggests that the drug acts on the liver cell mechanism for secreting substances into the bile. From observations of the effect of bromsulphthalein and indocyanine green on conjugation and biliary excretion Hargreaves and Lathe[19] suggested that two processes may be very closely linked. There are some indications that drugs affect the excretory process of cholephils in different ways, for instance, Hargreaves[20] has shown that male fern extract blocked the excretion of bilirubin but not that of indocyanine green. The effect of SKF 525-A on conjugation and excretion implies either a double site of action or that UDP glucuronyltransferase is linked very closely to the mechanism of excretion from the liver cell into the bile.

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