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INHIBITION OF β-GLUCURONIDASE BY 2-DIARYLMETHYL-1,3-INDANDIONES

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Abstract—The effects of several 2-diarylmethyl-1,3-indandiones on the activity of β-glucuronidase from different rat and bovine tissues was examined. 2-Benzhydryl-1,3-indandione appeared to be a potent inhibitor of β-glucuronidase whereas this compound did not affect some other lysosomal enzymes such as, acid phosphatase and some glycosidases. Small alterations of the molecular structure caused a loss of inhibitory activity. Introduction of substituents into the phenyl rings usually lowered the inhibitory activity and bulky substituents were very unfavourable. The inhibition could be correlated with physicochemical parameters, and both steric effects and lipophilicity appeared important. Electronic effects were of little consequence. The inhibition was uncompetitive and not related to anticoagulant activity.

In the course of our investigations on the effects of anti-inflammatory indandione derivatives on some biochemical processes [1, 2] we studied the effects on enzyme release from rat liver lysosomes [3]. None of the compounds investigated did affect this release but 2-benzhydryl-1,3-indandione (BHID) appeared to be an inhibitor of β-glucuronidase (EC 3.2.1.31). 1,3-Indandiones display several biological properties of which the anticoagulant and anti-inflammatory activities come most to the fore, and therefore, it would be of interest to study the inhibition of β-glucuronidase in more detail and, furthermore, its relation to other biological properties.

In vitro inhibition of β-glucuronidase has, until now, been reported for substrate analogues [4–7], acid mucopolysaccharides [8], heavy metal ions [6], a few lipids [19], peroxides [10] and some tissue fractions of unknown structure [6, 11, 12], but there was never a clear relation to the *in vivo* activities of these compounds. Phenylbutazone has likewise been reported to inhibit β-glucuronidase, albeit at rather high and unphysiological concentrations [13–15]. Inhibition by substrate analogues was of the competitive type, whereas in most other cases inhibition proved noncompetitive.

The present paper describes the effects of a series of substituted 2-diarylmethyl-1,3-indandiones and some related compounds on β-glucuronidase activity from different sources.

MATERIALS AND METHODS

Chemicals. 2-Diarylmethyl-1,3-indandiones and related compounds were synthesized as described previously [16]. Warfarin was obtained from Sigma Chemical Co, phenylbutazone from Ciba–Geigy and indomethacin from Merck Sharpe & Dohme. *p*-Nitrophenyl phosphate was obtained from BDH Chemicals Ltd., and the *p*-nitrophenyl glycosides used as substrates for the determination of the glycolytic enzymes listed in Table 1 were purchased from Koch–Light Laboratories Ltd. All other chemicals were analytical grade preparations from the usual sources.

Enzyme preparations. Two types of enzyme preparations were used, namely lysosome-rich fractions from different tissues of rat and calf and purified β-glucuronidase from rat or bovine liver. A lysosome-rich fraction from rat liver (Wistar strain) was prepared by centrifuging a 10% homogenate in 0·25 M sucrose at 900 *g* for 5 min and recentrifuging the supernatant at 25,000 *g* for 20 min. The pellet was suspended in 0·25 M sucrose and contained about 50 per cent of total β-glucuronidase activity and about 50 per cent of total protein. Lysosome-rich fractions from rat brain and kidney and calf liver, brain and kidney were prepared by the same method. β-Glucuronidase from rat liver was purified following the procedure of Stahl and Touster [17] up to and including the dialysis of the ammonium sulphate precipitate. The dialyzed materials of 10 rats were pooled and this preparation was used in most experiments. Purified β-glucuronidase from bovine liver was obtained from Sigma Chemical Co. (type B-1).

Protein content of each preparation was determined according to the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

Lysosomal enzyme assays. Acid phosphatase (EC 3.1.3.2) was determined according to the method of Torriani [19] using *p*-nitrophenyl phosphate as the substrate. β-Glucuronidase was determined by a modification of the method of Gianetto and De Duve [20] using *p*-nitrophenyl glucuronide as the substrate. The incubation medium (1 ml) contained 0·1 M acetate buffer, pH 4·5, 2·5 mM substrate, 0·03% (v/v) Triton X-100, 3% (v/v) ethanol and an appropriate amount of enzyme. After incubation at 37° the reaction was terminated by adding 4 ml of 0·1 M glycine/NaOH buffer, pH 10·3, and the absorbance of the nitrophenyl anion measured at 415 nm.

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Table 1. Effect of 0·1 mM BHID on various lysosomal enzymes present in the lysosome-rich fraction from rat liver

Enzyme	Incubation conditions		Enzyme activity	
	Buffer	pH	Control*	BHID present†
Acid phosphatase	acetate	4·5	143·0	96
α-Galactosidase	acetate	4·2	2·33	97
β-Galactosidase	citrate	4·3	6·94	98
α-Mannosidase	acetate	4·2	3·17	97
β-Glucosaminidase	citrate	4·3	34·9	103
β-Galactosaminidase	citrate	4·3	5·23	96
β-Glucuronidase	acetate	4·5	19·2	12
Neuraminidase**	acetate	4·0	0·28	101

Values are the means of two experiments.
* Activity as nmoles *p*-nitrophenol/min/mg protein.
† Activity as per cent of control.
** Neuraminidase was determined in grey matter from calf brain and activity expressed as nmoles neuraminic acid/min/mg protein.

The activities of some other glycosidases [α-galactosidase (EC 3.2.1.22), β-galactosidase (EC 3.2.1.23), α-mannosidase (EC 3.2.1.24), β-acetylglucosaminidase (EC 3.2.1.30) and β-acetylgalactosaminidase (EC 3.2.1.–)] were determined by similar procedures as described by Barrett [21] using *p*-nitrophenyl glycosides as the substrates. Acetate or citrate buffers of an appropriate pH were used as indicated in Table 1.

Neuraminidase (EC 3.2.1.18) was assayed with gangliosides as the substrate as described by Roukema and Heijlman [22].

All compounds tested were added to the incubation mixture as an ethanolic solution to a final ethanol concentration of 3% (v/v). This amount of ethanol did not influence enzyme activity.

Multiple regression analysis. Inhibitory activities have been correlated with physicochemical parameters using the Hansch approach [23]. π values were taken from Hansch [23], Hammett's electronic parameter (σ) from Jaffé [24] and the steric parameters from Taft [25]. Regression equations will be given under Results, where *n* is the number of compounds, *r* is the correlation coefficient, *s* is the standard error and *F* is the overall statistical significance of the equation. The Student's *t*-test values of the regression coefficients are placed in brackets.

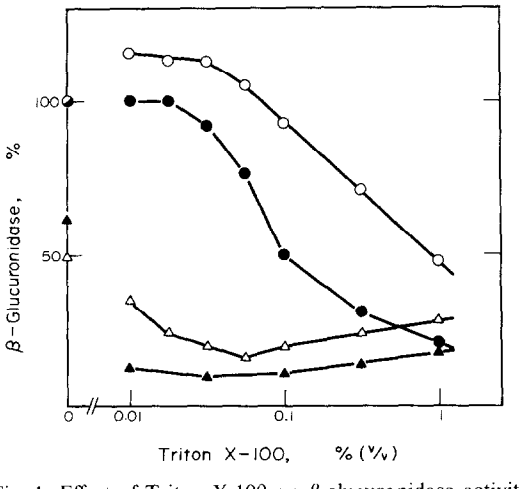


Fig. 1. Effect of Triton X-100 on β-glucuronidase activity in the lysosome-rich fraction from rat liver (○, control; △, 0·1 mM BHID present) and on the purified enzyme from rat liver (●, control; ▲, 0·1 mM BHID present).

RESULTS

Effect of BHID on β-glucuronidase. Figure 1 shows that BHID is an inhibitor of β-glucuronidase activity in rat liver. Since Triton X-100 is known to be an activator of lysosomal enzymes, the influence of this detergent on enzyme activity and inhibition by BHID was studied. Triton X-100 appeared to enhance the inhibitory action of BHID considerably but other studies showed that a Triton X-100 concentration of at least 0·01% is necessary to ensure the complete solubility of BHID under the incubation conditions used. Hence, in the experiments described in the present paper 0·03% Triton X-100 was always present in the incubation medium.

Specificity of inhibitory action. In order to establish whether the inhibitory action of BHID was specific for β-glucuronidase, the effects of this drug on some other lysosomal enzymes including some glycosidases, were studied. Table 1 shows that only β-glucuronidase is inhibited by BHID. This inhibition is, however, not restricted to β-glucuronidase from rat liver but also occurs in other rat tissues and in some bovine tissues (see Table 2).

Inhibition by derivatives of BHID. The effects of a series of substituted derivatives of BHID on purified β-glucuronidase from rat liver were studied. Inhibition was measured at three suitable drug concentrations and the concentration producing 50 per cent inhibition (C_{50}) was determined graphically. The C_{50} values listed in Table 3 are the means of five experiments with S.D.

The activities of two derivatives substituted in the indandione ring and a few analogues derived from 4-hydroxycoumarin were also studied and the results are shown in Table 4.

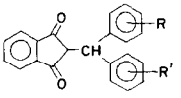
The activities of some compounds chemically related to BHID were evaluated so as to ascertain on what structural elements of the BHID molecule

Table 2. Effect of BHID on β-glucuronidase activity in lysosome-rich fractions from different sources and on the purified enzyme

	Enzyme activity			
		Activity in the presence of varied concentrations of BHID (μM) as % of control		
Source	Control*	1	10	100
Rat liver	19·1 ± 5·0	95 ± 1	43 ± 7	14 ± 3
Rat kidney	4·35 ± 1·35	86 ± 3	36 ± 1	6 ± 1
Rat brain	0·51 ± 0·10	100 ± 1	88 ± 1	58 ± 4
Calf liver	10·9 ± 2·1	94 ± 2	75 ± 5	28 ± 2
Calf kidney	2·70 ± 0·50	93 ± 2	62 ± 4	13 ± 3
Calf brain	0·28 ± 0·07	100 ± 2	90 ± 1	65 ± 4
Rat liver purified	110 ± 5	86 ± 3	23 ± 2	13 ± 2
Bovine liver purified	173 ± 2	76 ± 2	45 ± 2	37 ± 1

Values are the means of 5 experiments with S.D.
* Activity as nmoles *p*-nitrophenol/min/mg protein.

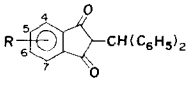
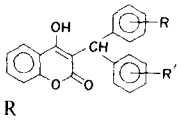
Table 3. Effects of 2-diarylmethyl-1,3-indandiones on purified β-glucuronidase from rat liver.



Compound No.	R	R'	C_{50} μM
1	H	H	4·8 ± 0·7
2	4-methyl	H	6·6 ± 0·2
3	4-ethyl	H	7·6 ± 0·6
4	4-t-butyl	H	10·2 ± 1·1
5	4-fluoro	H	7·2 ± 0·6
6	4-chloro	H	8·8 ± 1·3
7	4-bromo	H	9·8 ± 0·9
8	4-dimethylamino	H	> 200
9	4-methyl	4-methyl	10·9 ± 1·4
10	4-t-butyl	4-t-butyl	32·5 ± 6·6
11	4-t-butyl	4-fluoro	17·5 ± 1·9
12	4-fluoro	4-fluoro	9·8 ± 1·0
13	3-methyl	H	3·6 ± 0·3
14	3-t-butyl	H	7·3 ± 1·1
15	3-chloro	H	6·4 ± 0·9
16	3,5-di-t-butyl	H	> 200
17	3,5-di-t-butyl-4-hydroxy	H	> 200
18	3,5-dimethyl	3,5-dimethyl	7·9 ± 0·1
19	2-methyl	H	9·0 ± 0·9
20	2-ethyl	H	7·1 ± 0·2
21	2-isopropyl	H	33·0 ± 3·8
22	2-t-butyl	H	39·2 ± 4·0
23	2-chloro	H	11·1 ± 0·7
24	2-methyl	2-methyl	16·7 ± 0·8
25	2-ethyl	2-ethyl	37·2 ± 2·9
26	2-isopropyl	2-isopropyl	122 ± 18
27	2-t-butyl	2-t-butyl	> 200
28		2,2'-ethylene*	9·0 ± 1·5
29		2,2'-vinylene†	> 200
30	2,6-dimethyl	H	69 ± 8
31	2,6-diisopropyl	H	> 200
32	2,6-dichloro	H	> 200
33	2,6-dimethyl	2-methyl	95 ± 11
34	2,6-dichloro	2-methyl	> 200
35	2,6-dimethyl	2,6-dimethyl	> 200
36	2,6-dichloro	2,6-dimethyl	> 200

Results are the means of 5 experiments with S.D.
* 2-(10,11-Dihydro-5H-dibenzo[a,d]cyclohepten-5-yl)-1,3-indandione.
† 2-(5H-Dibenzo[a,d]cyclohepten-5-yl)-1,3-indandione.

Table 4. Effects of some BHID derivatives and 4-hydroxycoumarin analogues on purified β-glucuronidase from rat liver.

No.	Compound		C_{50} (μM)
			
37	R = 4-nitro		7·3 ± 1·0
38	R = 4,5,6,7-tetrachloro		3·6 ± 0·7
			
	R	R′	
39	H	H	61 ± 10
40	4-methyl	4-methyl	87 ± 12
41	4-fluoro	4-fluoro	111 ± 25
42	2,6-dimethyl	2,6-dimethyl	> 200

Results are the means of 5 experiments with S.D.

activity would be dependent. Table 5 shows the inhibitions produced by 0·1 mM of these compounds (means of three experiments).

The C_{50} values of the active compounds listed in Table 3 were analyzed using the Hansch approach [23] and the physicochemical parameters used are listed in Table 6. Compound 28 was omitted as the parameters for this substitution pattern were not available. Compared with the 2,2′-disubstituted compounds, the 2,6-disubstituted derivatives form a particular group which did not fit in the regression (see Discussion). Hence, compounds 30 and 33 were omitted from the calculations.

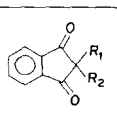
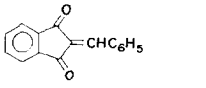
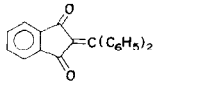
For the whole series of compounds listed in Table 6 eq. 1 was obtained:

$$-\log C_{50} = 4{\cdot}920 - 0{\cdot}419\,\pi_o + 0{\cdot}134\,E_s^p \qquad (1)$$
$$(-13{\cdot}691)\ (7{\cdot}157)$$
$$n = 23 \quad r = 0{\cdot}952 \quad s = 0{\cdot}115 \quad F = 95{\cdot}74$$

where π_o is the sum of π values of the *ortho* substituents and E_s^p the steric parameter for *para* substituents. It follows from this equation that substitution into *meta* position exerts no effect on the inhibitory activity.

For *para*-substituted compounds (Nos 1–12) eq. 2 was found and the activities of the *ortho*-substituted

Table 5. Effects of some 1,3-indandione derivatives on purified β-glucuronidase from rat liver.

No.	 R_1	R_2	β-glucuronidase activity in the presence of 0·1 mM drug as per cent of control
43	C_6H_5	H	97
44	$CH_2C_6H_5$	H	95
1	$CH(C_6H_5)_2$	H	11
45	$C(C_6H_5)_3$	H	96
46	$COCH(C_6H_5)_2$	H	88
47	$CH(C_6H_5)_2$	NO_2	101
48			99
49			98
50	$HOOC\text{-}CH_2CH(C_6H_5)_2$		92
51	Warfarin		93
52	Phenylbutazone		74
53	Indomethacin		98

Results are the means of 3 experiments.

Table 6. Inhibitory activities and physicochemical properties of some 2-diarylmethyl-1,3-indandiones.

Compound No.	π_o*	σ†	E_s^p‡	$-\log c_{50}$ (M^{-1}) Found	Calc.**
1	0·00	0·00	2·48	5·32	5·25
2	0·00	−0·17	1·24	5·18	5·09
3	0·00	−0·15	1·17	5·12	5·08
4	0·00	−0·20	−0·30	4·99	4·88
5	0·00	0·06	2·02	5·14	5·19
6	0·00	0·23	1·51	5·06	5·12
7	0·00	0·23	1·32	5·01	5·10
9	0·00	−0·34	0·00	4·96	4·92
10	0·00	−0·40	−3·08	4·49	4·51
11	0·00	−0·14	−0·76	4·76	4·82
12	0·00	0·12	1·56	5·01	5·13
13	0·00	−0·07	2·48	5·44	5·25
14	0·00	−0·10	2·48	5·14	5·25
15	0·00	0·37	2·48	5·19	5·25
18	0·00	−0·28	2·48	5·10	5·25
19	0·56		2·48	5·05	5·02
20	1·02		2·48	5·15	4·83
21	1·53		2·48	4·48	4·61
22	1·98		2·48	4·41	4·42
23	0·71		2·48	4·96	4·96
24	1·12		2·48	4·78	4·78
25	2·04		2·48	4·43	4·40
26	3·06		2·48	3·91	3·97

* From ref. 23; † from ref. 24; ** by using eq. 1; ‡ from ref. 25.

derivatives (Nos 19–26) showed a good correlation with π_o alone (eq. 3):

$$-\log c_{50} = 4{\cdot}863 + 0{\cdot}172\,E_s^p - 0{\cdot}419\,\sigma \quad (2)$$
$$(11{\cdot}126) \quad (-3{\cdot}656)$$
$$n = 11 \quad r = 0{\cdot}977 \quad s = 0{\cdot}053 \quad F = 82{\cdot}84$$
$$-\log c_{50} = 5{\cdot}362 - 0{\cdot}476\,\pi_o \quad (3)$$
$$(-7{\cdot}783)$$
$$n = 8 \quad r = 0{\cdot}954 \quad s = 0{\cdot}135 \quad F = 60{\cdot}58$$

Calculations in which other parameters, such as E_s^o (steric parameter for *ortho* substituents), π_{mp} (sum of the π values of *meta* and *para* substituents) and π_{tot} (sum of the π values of all substituents) were used provided equations of poor quality.

Mechanism of inhibitory action. Further studies concerning the inhibition of β-glucuronidase by BHID showed that inhibition was not time-dependent and constant over a pH-range from 3·6–5·5. At pH 7·4 (0·1 M sodium acetate) the inhibition was, however, much lower.

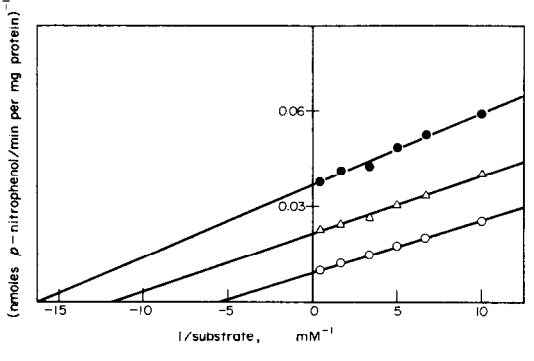


Fig. 2. Lineweaver–Burk plot of the inhibition of purified β-glucuronidase from rat liver by BHID: ○, control; △, 5 μM BHID and ●, 10 μM BHID.

The enzyme reaction shows the normal Michaelis–Menten kinetics, and in the absence of inhibitor a K_m value of 0·16 mM was found. This is in agreement with the value of 0·12 mM reported by Fishman *et al.* [26]. However, inhibition appeared to be dependent on the substrate concentration, and the Lineweaver–Burk plot showed that the inhibition was of the uncompetitive type (Fig. 2).

DISCUSSION

2-Diarylmethyl-1,3-indandiones have anticoagulant properties which result from the inhibition of the biosynthesis of vitamin K-dependent clotting factors in the liver [16]. The inhibition of β-glucuronidase described in the present paper is another interesting property of these compounds which is, apparently, not related to anticoagulant activity as 2-phenyl-1,3-indandione (43), 2-diphenylacetyl-1,3-indandione (46) and warfarin (51) which are potent anticoagulant agents, have no effect on β-glucuronidase. Inhibition of β-glucuronidase by phenylbutazone (52) has been reported more than once [13–15] but in spite of these findings it is not very likely that there is any relationship between this inhibition and anti-inflammatory action as indomethacin (53) does not affect β-glucuronidase (see Table 5) and BHID is devoid of anti-inflammatory activity [16]. Since BHID already exhibits a significant inhibition of β-glucuronidase at 1–10 μM, this effect might have some significance under physiological conditions. In this connection, it would be of interest to investigate if BHID can inhibit the development of bladder tumours, a property

which has been reported for β-glucuronidase inhibitor SLA [27, 28].

The inhibitory effect of BHID on β-glucuronidase is rather specific: small alterations in the molecule eliminate activity (see Table 5) and, furthermore, some related enzymes are not affected by this compound. On the other hand, all enzyme preparations investigated could be inhibited by BHID (Table 2). The effects on liver and kidney enzymes are comparable but the enzyme from brain is less sensitive to BHID. Furthermore, enzymes from rat tissues are inhibited more strongly than those from bovine tissues. The differences might be due to different characteristics of the enzymes (tissue and species differences of β-glucuronidase have been reported by Levvy and Conchie [6]) but some caution must be used in the interpretation of these results. The specific activity of β-glucuronidase in the lysosomal fraction from brain is rather low which implies the presence of many other proteins. These accompanying proteins possibly bind BHID, thus lowering the effective concentration of this compound. This causes an apparently poor activity of BHID on β-glucuronidase from brain. This view is supported by the fact that the purified liver enzymes are inhibited in a more pronounced way than the enzymes present in the lysosome-rich fractions from liver (Table 2).

As can be seen in Table 3, there exists a clear relationship between inhibitory activity and the structure of a series of BHID derivatives. The unsubstituted compound (BHID) is a strong inhibitor and, generally speaking, substitution lowers activity. Steric effects or lipophilicity appear to play a major role and substitution with three or four *ortho* substituents or substitution with bulky groups causes a loss of activity (see compounds 27, 31 36). NMR studies of these compounds revealed that compounds 28–34 have an anti conformation with respect to the vicinal aliphatic protons, whereas all other compounds show a gauche conformation [16]. The great difference in activity between compounds 28 and 29 is however not clear, as these compounds have the same molecular conformation and about the same lipophilicity.

Multiple regression calculations carried out with the compounds listed in Table 6 confirm the above considerations. Substitution by lipophilic or bulky groups into *ortho* or *para* position lowers activity considerably whereas electronic effects are of little consequence (eq. 1–3). It should be noted that the activities of too few *meta*-substituted compounds have been determined for a reliable assessment of the effect of *meta* substitution on the inhibitory activity. Lack of activity of compounds 16 and 17 shows, however, that the introduction of two bulky *meta* substituents into the same phenyl ring causes a loss of activity. This indicates that in the *meta* series steric effects probably also play an important role, which does not appear from eq. 1. Similarly, the low activity of compound 8 is not predicted by eq. 2 ($E_s^p = 0{\cdot}77$ and $\sigma = -0{\cdot}83$) but the presence of the basic amino group may be decisive in this respect. On the contrary, eqs 1 and 3 do predict the low activity of compound 27 ($\pi_o = 3{\cdot}96$). Substitution into the indandione moiety scarcely affects the inhibitory action, suggesting that this part of the molecule is less important (see Table 4). The 4-hydroxycoumarin derivatives investigated are much less active than their 1,3-indandione analogues, although in both series the effects of substitution in the two phenyl rings are about the same.

Table 5 shows that any change in the BHID molecule causes loss of activity and it can be concluded that two phenyl rings and, moreover, two vicinal aliphatic protons are essential for good activity. The indandione part is also necessary being that the 4-hydroxycoumarin analogue of BHID is only slightly active and compound 50 totally inactive.

The mechanism of the inhibitory action of BHID has not yet been elucidated. In contrast to substrate analogues which are competitive inhibitors of β-glucuronidase [6, 7] and some tissue factors which are noncompetitive inhibitors [11, 12], BHID causes an inhibition of the uncompetitive type (Fig. 2). This is a rather unusual type of inhibition but has also been reported by Tappel and Dillard for the inhibition of β-glucuronidase by cholesterol [9]. These authors believe that the inhibition by cholesterol is due to a disaggregation of the enzyme molecule into inactive units. Other studies by Christner *et al.* [10] showed that β-glucuronidase is not inhibited by cholesterol but by the hydroperoxide formed during autoxidation. Other peroxides, too, proved to be inhibitors and the activity increased in accordance with the lipophilic character of the compounds. This is rather in contrast to the results described in the present paper for the 2-diarylmethyl-1,3-indandiones, where the increase in lipophilicity has a negative effect on the inhibitory activity (eqs 1–3). Since drug protein binding has been reported to increase with lipophilicity [29], it is not very likely that the inhibitory action of the 2-diarylmethyl-1,3-indandiones results from binding of these compounds to the protein part of β-glucuronidase. In addition, the inhibition of β-glucuronidase by BHID is of the uncompetitive type which might point to a mechanism in which the inhibitor binds to the enzyme-substrate complex. However, further experiments will be necessary to elucidate the mechanism of β-glucuronidase inhibition by 2-diarylmethyl-1,3-indandiones.

Note added in proof: Recently, Marselos *et al.* reported that the β-glucuronidase inhibitor D-glucaro-1,4-lactone can shorten the pharmacological action of drugs like phenobarbital and progesterone by enhancing the excretion of their glucuronic acid conjugates [M. Marselos, G. Dutton and O. Hänninen, *Biochem. Pharmac.* **24**, 1855 (1975)]. This effect may also be important in connection with the *in vivo* effects of the 2-diarylmethyl-1,3-indandiones described in the present paper.

REFERENCES

1. G. van den Berg and W. Th. Nauta, *Biochem. Pharmac.* **24**, 815 (1975).
2. G. van den Berg, T. Bultsma and W. Th. Nauta, *Biochem. Pharmac.* **24**, 1115 (1975).
3. G. van den Berg, 2-Aryl-1,3-indandiones. Ph.D. Thesis, Vrije Universiteit, Amsterdam (1974).
4. G. A. Levvy, A. McAllan and C. A. Marsch, *Biochem. J.* **69**, 22 (1958).
5. R. Iida, S. Nagata, M. Kakimoto, H. Akaike, H. Watanabe and A. Shioya, *Jap. J. Pharmac.* **15**, 88 (1965).
6. G. A. Levvy and J. Conchie, in *Glucuronic Acid* (Ed. G. J. Dutton) p. 301. Academic Press, New York (1966).

7. T. Niwa, T. Tsuruoka, S. Inouye, Y. Naito, T. Koeda and T. Niida, *J. Biochem. Tokyo* **72,** 207 (1972).
8. P. G. Walker and G. A. Levvy, *Biochem. J.* **54,** 56 (1953).
9. A. L. Tappel and C. J. Dillard, *J. biol. Chem.* **242,** 2463 (1967).
10. J. E. Christner, S. Nand and N. S. Mhatre, *Biochem. biophys. Res. Commun.* **38,** 1098 (1970).
11. W. Sakamoto, O. Nishikaze and E. Sakakibara, *J. Biochem. Tokyo* **75,** 675 (1974).
12. W. Sakamoto, O. Nishikaze and E. Sakakibara, *Biochim. biophys. Acta* **343,** 409 (1974).
13. J. H. Brown and N. L. Schwartz, *Proc. Soc. exp. Biol. Med.* **131,** 614 (1969).
14. J. O. Malbica and L. G. Hart, *Biochem. Pharmac.* **20,** 2017 (1971).
15. L. J. Ignarro, *Biochem. Pharmac.* **20,** 2847 (1971).
16. M. L. de Winter, Ph.D. Thesis, Vrije Universiteit, Amsterdam (1975).
17. P. D. Stahl and O. Touster, *J. biol. Chem.* **246,** 5398 (1971).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
19. A. Torriani, *Biochim. biophys. Acta* **38,** 460 (1960).
20. R. Gianetto and C. de Duve, *Biochem. J.* **59,** 433 (1955).
21. A. J. Barrett, in *Lysosomes—A Laboratory Handbook* (Ed. J. T. Dingle) p. 116. North-Holland Publ. Comp. Amsterdam (1972).
22. P. A. Roukema and J. Heijlman, *J. Neurochem.* **17,** 773 (1970).
23. C. Hansch, in *Structure–Activity Relationships* (Ed. C. J. Cavallito) Vol. I, p. 75. Pergamon Press, Oxford (1973).
24. H. Jaffé, *Chem. Rev.* **53,** 191 (1953).
25. R. W. Taft, in *Steric Effects in Organic Chemistry* (Ed. M. S. Newman), p. 644. Wiley, New York (1956).
26. W. H. Fishman, Y. Nakajima, C. Anstiss and S. Green, *J. Histochem. Cytochem.* **12,** 298 (1964).
27. M. Miyakawa, T. Harada, O. Yoshida and T. Kato, *Acta urol. jap.* **16,** 738 (1970).
28. M. Miyakawa, O. Yoshida, T. Harada and T. Kato, *Invest. Urol.* **10,** 256 (1973).
29. C. Hansch and W. J. Dunn, *J. pharm. Sci.* **61,** 1 (1972).