Intrinsic and Intramolecular Lipophilicity Effects in *O*-Glucuronides

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In this study, we compared the lipophilicity of *O*-glucuronides and their aglycones. Distribution coefficients (log *D*) and *P* values of neutral species (log *P*) were determined by centrifugal partition chromatography (CPC) in octanol/buffer systems. Two-phase potentiometry was also used to measure the log *P* value of some lipophilic solutes. The experimentally determined global influence of glucuronidation on lipophilicity, obtained as the difference (decrement) log $P_{(glucuronide)}$ – log $P_{(aglycone)}$, was found to be -1.30 ± 0.16 ($n = 4$) for glucuronides of alcohols (methyl, menthyl, neomenthyl, and chloramphenicol *O*-glucuronide). The mean decrement was -2.06 ± 0.31 ($n = 9$) for glucuronides of phenols (phenyl, *p*-nitrophenyl, 1-naphthyl, 6-bromo-2-naphthyl, 4-methylumbelliferyl, 3-coumarinyl, phenolphthalein, 4'-benzophenonyl *O*-glucuronide, and diflunisal phenolic glucuronide). For the acylglucuronide of diflunisal and its rearrangement isomers, the mean decrement was -1.80 ± 0.08 ($n = 4$; range -1.7 to -1.9). Differences in through-bond proximity effects as parametrized in the CLOGP algorithm seem to account for much of this difference. Conformational factors may also play a role, although it appears modest and unassessable for the glucuronides investigated here. The results imply that *in vivo* glucuronidation should have a stronger influence on the excretion of phenols than on that of alcohols.

1. Introduction. – Glucuronidation is a major conjugation pathway in the metabolism of drugs and other xenobiotics. Glucuronides are considered as highly polar metabolites characterized by a limited capacity to cross biological membranes (in particular the blood-brain barrier) and a rapid urinary or biliary excretion [1–7]. However, little quantitative information exists on the lipophilicity of glucuronides as compared to that of substrates (*i.e.*, aglycones). First argument in this respect is the influence of glucuronidation on ionization. Considering only the ionizability of the moiety being glucuronidated, the following cases have been recognized [2][4]:

- O-*glucuronidation of alcohols*: addition of the strong acidic group of the glucuronyl moiety;
- O-*glucuronidation of phenols and* S-*glucuronidation of thiols*: replacement of a weakly acidic group with the strong acidic group of the glucuronyl moiety;
- O-*glucuronidation of carboxylic acids and* C-*glucuronidation of C-acids*: replacement of a strong acidic group with the strong acidic group of the glucuronyl moiety;
- N-*glucuronidation of dimethylamino groups*: replacement of a basic tertiary amino group with a quaternary ammonium group, and addition of the strong acidic group of the glucuronyl moiety.

Such considerations, however, remain qualitative. To the best of our knowledge, no systematic study has been undertaken to quantify the changes in lipophilicity resulting

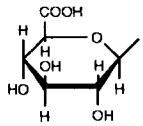
from glucuronidation, and to look for intramolecular effects that could influence these changes.

That such changes can be operative has been suggested for morphine, whose conjugation in humans and animals yields morphine-3-*O*-β-D-glucuronide (M3G) and morphine-6-*O*-β-D-glucuronide (M6G). A number of studies have concluded that M3G and M6G have a genuine capacity to penetrate the brain [8–14], whereas others have not been so conclusive [15][16]. What is clear, however, is the remarkable analgesic activity of M6G, which, when administered peripherally to animals and patients, proved to be more potent and longer acting than morphine [10][17–21]. Such *in vivo* effects result from a high affinity for the opiate receptor [22–25], but they also imply brain penetration. As for M3G, it has been suggested to be a weak antagonist of morphine *in vivo* [26–28].

These results prompted an experimental and computational study of the lipophilicity of morphine and its glucuronides [29]. The results obtained by RP-HPLC showed that these glucuronides are not as hydrophilic as expected, and in fact about one order of magnitude (on the log k scale) less polar than morphine. This property has been explained in part by a 'chameleonic' behavior of M6G and M3G so that they can exist in both extended, hydrophilic conformations, and in folded, more lipophilic conformations [29].

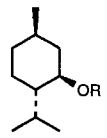
The present study is a continuation of the work on M3G and M6G, focusing mainly on glucuronides of alcohols and phenols, *i.e.*, ether glucuronides whose only ionizable group was the carboxyl function of glucuronic acid. Our first goal was to quantify the contribution of the *O*-glucuronyl fragment to the log P value of such glucuronides. The second goal was to search for intramolecular factors influencing the lipophilicity of glucuronides. To these ends, we measured and compared the octanol/H_2O lipophilicity of various *O*-glucuronides and their aglycones using centrifugal partition chromatography (CPC) and pH-metry. The antiinflammatory drug diflunisal was also of interest since it forms both a phenolic (ether) and an acyl (ester) glucuronide [30][31]. The latter, like other acyl glucuronides, is chemically reactive *in vitro* and *in vivo*, undergoing hydrolysis, rearrangement (*via* acyl migration of the drug moiety to yield the 2-, 3-, and 4-isomers), and covalent binding reactions with proteins [32]. The covalent binding is attracting interest as a possible mechanism (haptenization) explaining hypersensitivity responses to acidic drugs [33][34]. Diflunisal acyl glucuronide and its 2-, 3-, and 4-isomers show quite different capacities to covalently bind diflunisal to human serum albumin [35]. These four isomeric structures were examined in the present study to gain a first insight into the lipophilicity of ester glucuronide isomers.

2. Results and Discussion. – 2.1. *p*K_a *Values.* The pK_a values of the glucuronides **1**–**14** (as well as those of a few aglycones, results not shown) were determined and are presented in the *Table*. The value of glucuronic acid was found to be 3.00 in agreement with the literature [36]. With few exceptions, the pK_a values of the glucuronides are close to 3, the differences being too small to be meaningful. The available amounts of diflunisal glucuronides were too limited to allow reliable pK_a determination. The first pK_a of diflunisal is 3.00 [36], and its second can be estimated to be close to 12 by analogy with salicylic acid.

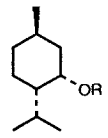


β-D-glucuronyl (=gluc)

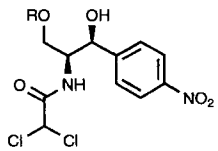
1a R = H
1g R = gluc



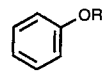
2a R = H
2g R = gluc



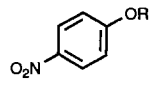
3a R = H
3g R = gluc



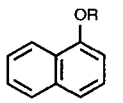
4a R = H
4g R = gluc



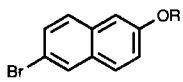
5a R = H
5g R = gluc



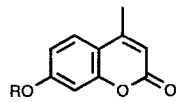
6a R = H
6g R = gluc



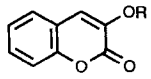
7a R = H
7g R = gluc



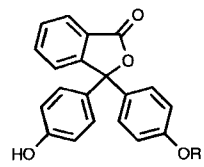
8a R = H
8g R = gluc



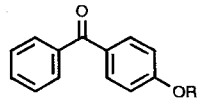
9a R = H
9g R = gluc



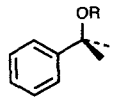
10a R = H
10g R = gluc



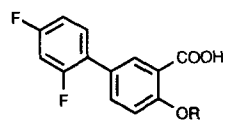
11a R = H
11g R = gluc



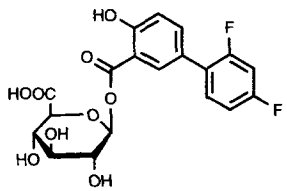
12a R = H
12g R = gluc



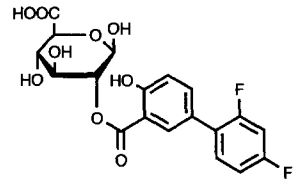
13a R = H
13g R = gluc



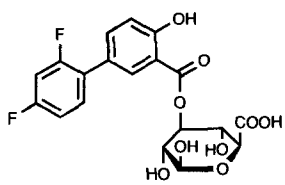
14a R = H
14g-ether R = gluc



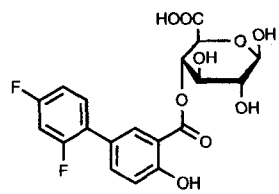
14g-1-ester



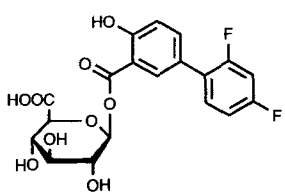
14g-2-ester



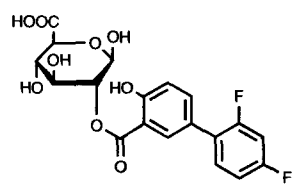
14g-3-ester



14g-4-ester



14g-1-ester



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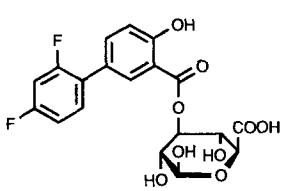

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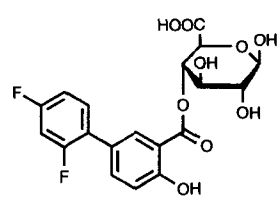

14g-4-ester

2.2. *Distribution and Partition Coefficients (*log *D* and log *P*, Respectively). The *Table* compiles the log *P* values obtained for the aglycones. Most values were measured directly by CPC. For the acidic aglycones, log *D* values were corrected for ionization using *Eqn. 3* (*Exper. Part*). Two-phase titration proved the method of choice to obtain the log *P* value of diflunisal. Experimental difficulties prevented obtaining values for compounds **2a**, **3a** (detection problems), and **8a** (too high lipophilicity), whereas compound **10a** was not commercially available; the log *P* value of these solutes was, therefore, calculated using CLOGP [37]. The values of compounds **7a** and **12a** were taken from the literature [36].

Table. pK_a *and log* P *Values of Glucuronides and Their Aglycones*

Compounds	pK_a of glucuronide (series **g**)	log *P* of aglycone (series **a**)[a])	log *P* of glucuronide (series **g**)[a])	log $P_{(glucuronide)}$ – log $P_{(aglycone)}$[b])	CLOGP of aglycones (series **a**)	CLOGP of glucuronides (series **g**)
Glucuronic acid	3.00 ± 0.08		−2.55 ± 0.02			
1 (**a** or **g**)	2.98 ± 0.03	−0.68 ± 0.02	−2.22 ± 0.07	−1.5	−0.76	−2.89
2 (**a** or **g**)	3.17 ± 0.07	3.23[c])	1.96 ± 0.02	−1.3	3.23	1.11
3 (**a** or **g**)	3.08[d])	3.23[c])	2.16 ± 0.04	−1.1	3.23	1.11
4 (**a** or **g**)	2.95 ± 0.02	1.18 ± 0.02	−0.17 ± 0.04	−1.3	0.69	−1.40
5 (**a** or **g**)	2.98 ± 0.01	1.49 ± 0.02	−0.46 ± 0.05	−1.9	1.47	−1.20
6 (**a** or **g**)	2.87 ± 0.03	1.98 ± 0.11	−0.24 ± 0.04	−2.2	1.85	−1.14
7 (**a** or **g**)	2.97 ± 0.04	2.84[d])	1.09 ± 0.09	−1.7	2.65	−0.01
8 (**a** or **g**)	2.92 ± 0.13	3.74[c])	1.94 ± 0.02	−1.8	3.74	0.96
9 (**a** or **g**)	2.96 ± 0.03	1.98 ± 0.05	−0.42 ± 0.02	−2.4	2.11	−1.00
10 (**a** or **g**)	2.87[d])	1.90[c])	−0.23 ± 0.03	−2.1	1.90	−1.37
11 (**a** or **g**)	3.04[d]) 9.09[d])	3.17 ± 0.05[e])	1.45 ± 0.08	−1.7	3.67	1.01
12 (**a** or **g**)	2.90[d])	3.07[f])	0.97 ± 0.10	−2.1	3.05	0.16
13 (**a** or **g**)	3.21[d])	1.69 ± 0.11	−0.03 ± 0.04	−1.7	1.81	−0.31
14g-ether	n.d.[g])	4.25 ± 0.05[e])	1.66 ± 0.02	−2.6	4.40	0.54
14g-1-ester	n.d.	4.25 ± 0.05[e])	2.36 ± 0.05	−1.9	4.40	1.37
14g-2-ester	n.d.	4.25 ± 0.05[e])	2.52 ± 0.06	−1.7	4.40	1.49
14g-3-ester	n.d.	4.25 ± 0.05[e])	2.43 ± 0.05	−1.8	4.40	1.47
14g-4-ester	n.d.	4.25 ± 0.05[e])	2.42 ± 0.05	−1.8	4.40	1.49

[a]) Obtained from log *D* values usually measured at two pH values in the range 2–7. [b]) Rounded to the first decimal. [c]) Calculated by the CLOGP algorithm [37]. [d]) Single measurement due to limited amount available. [e]) Determined by two-phase titration (*PCA 101* instrument). [f]) Taken from [36]. [g]) Not determined.

The log *D* values of the glucuronides were usually measured at two slightly acidic pH values chosen so that sufficient neutral form was present but no product of hydrolysis could be detected by CPC. The glucuronides of diflunisal could only be investigated at pH 2 due to peak broadening, but no free diflunisal was detected under the conditions of study. *Eqn. 1* or *2* was used to correct for ionization and obtain log *P* values. It must be stressed that the values for glucuronides listed in the *Table* refer to the neutral forms of the compounds.

2.3. *Changes in Lipophilicity Resulting from Glucuronidation.* The main objective of this work was to gain insight into some of the factors affecting the lipophilicity of glucuronides. First step in interpreting the experimental data compiled in the *Table* was,

therefore, to express in quantitative terms the change (decrement) in lipophilicity resulting from glucuronidation of a given substrate. This was done by calculating log $P_{(glucuronide)} - \log P_{(aglycone)}$. The values of this parameter (*Table*) vary between -1.1 and -2.6. A difference is evident between the glucuronides of alcohols ($n = 4$; range -1.1 to -1.5; mean -1.30 ± 0.16) and phenols ($n = 9$; range -1.7 to -2.6; mean -2.06 ± 0.31). The only exception is (dimethyl)(phenyl)methyl β-D-glucuronide **13g**, which showed a change in log *P* of -1.7, a value intermediate between those of alkyl and aryl glucuronides. Interestingly, this compound is the only benzyl-alcohol derivative in the series.

For the acylglucuronide of diflunisal and its isomers, the mean influence of glucuronidation was -1.80 ± 0.08 ($n = 4$; range -1.7 to -1.9). The acylglucuronide of diflunisal are less hydrophilic than diflunisal phenolic glucuronide (*Table*), whereas the differences in hydrophilicity between the acylglucuronide isomers were quite small. This shows that the considerable differences achieved in retention of these isomeric acylglucuronides on RP-HPLC [38] are due to specific interactions with the eluent and stationary phase more than to any intrinsic differences in lipophilicity.

2.4. *Assessment of Intramolecular Effects.* To understand the intramolecular interactions accounting for the different lipophilicity decrements, all glucuronides in this study were investigated for the possibility of 3D (conformational) or 2D (through-bond) effects on their lipophilic characteristics.

Conformational Behavior of Alkyl and Aryl Ether Glucuronides and Virtual log P *Ranges.* As already demonstrated [39–41], 3D effects on lipophilicity can be assessed by using the molecular lipophilicity potential (MLP) to calculate *virtual* log P *values* for all conformers obtained by a high-temperature quenched molecular dynamics exploration of the conformational space. This was done for all glucuronides in this study.

The aglycone fragments were characterized by a low or very low flexibility. In contrast, the glucuronyl fragment always displayed a marked conformational freedom, its main classes of conformers being:

- chair conformers with the COOH, OH, and *O*-aglyconyl substituents in equatorial positions (C_{eq} conformers);
- chair conformers with these substituents in axial positions (C_{ax} conformers);
- twist-boat conformers (TB conformers).

These conformers had markedly different internal H-bonding patterns. Furthermore, the C_{eq} conformers allowed the greatest distance between the glucuronyl and aglycone moieties (extended conformations), whereas the C_{ax} conformers were characterized by an intramolecular proximity between the two moieties (folded conformations). Also, the C_{ax} conformers of arylglucuronides were characterized by a stacking interaction between the aromatic part and the carboxylic group of the glucuronyl moiety. The TB conformers had intermediate internal distances between the two moieties. *Fig. 1* illustrates these features using representative conformers of menthyl β-D-glucuronide **2g** and 6-bromo-2-naphthyl β-D-glucuronide **8g** as examples. The differences in energy between the various classes of conformers were too modest (a few kcal/mol at the most) to allow any conclusion regarding conformational preferences in solution. Also, the calculations did not reveal any clear-cut difference between the conformational behavior of alkylglucuronides and arylglucuronides.

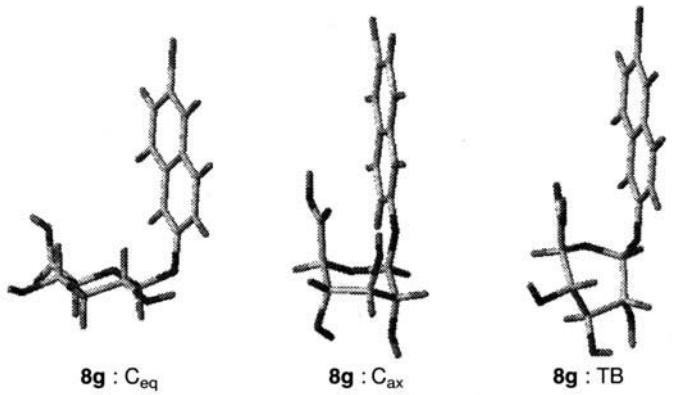

Fig. 1. *Representative conformers of 6-bromo-2-naphthyl β-D-glucuronide* (**8g**) *as obtained by quenched molecular dynamics.* C_{eq}: Chair conformers with the COOH, OH, and O-aglycone substituents in an equatorial position; C_{ax}: chair conformers with these substituents in an axial position; TB: twist-boat conformers.

Having obtained information on the conformational behavior of *O*-glucuronides, the next step was to search for differences in lipophilicity and 3D lipophilicity between individual conformers. However, the MLP in its present state of development is not adequately parametrized to calculate the log *P* of carbohydrates. Thus, a lipophilicity range between −3.94 and −3.62 and between −1.57 and −1.40 was calculated for glucuronic acid using the hydrophobic atomic increments of *Broto et al.* [42] and *Ghose* and coworkers [43], respectively, whereas the experimental value is −2.55 (*Table*). For this reason, only relative log *P* values obtained with the increments of *Broto et al.* are considered below.

The range of virtual log *P* values (*i.e.*, the difference in virtual log *P* between the most hydrophilic and lipophilic conformers of each compound) was generally limited and did not differ for alkyl and aryl glucuronides. This range was 0.3 for glucuronic acid and compounds **1g**, **5g**, **6g**, **7g**, **9g**, and **13g**; 0.4 for compounds **2g**, **3g**, **8g**, **11g**, and **12g**; 0.5–0.6 for compounds **10g** and **14g**; and 0.9 for compound **4g**. For comparison, the lipophilicity range of M6G and M3G was 0.6 and 0.8, respectively, with only two classes of conformers existing [29].

For all glucuronides, the more hydrophilic conformers were generally extended ones (C_{eq} conformers), whereas the more lipophilic conformers were folded and twist-boat ones (C_{ax} and TB conformers). Variations in the intramolecular H-bonding pattern of the glucuronyl moiety, as well as mutual masking of the glucuronyl (polar) and aglycone (hydrophobic) moieties, seemed to account for these differences in virtual lipophilicity. However, the comparable conformational behavior and the rather limited lipophilicity range of the various glucuronides did not reveal differences between aryl and alkyl glucuronides. Thus, the differences in experimental lipophilicity between alkyl and aryl glucuronides could not be explained by 3D (conformational) effects.

Search of 2D-Proximity Effects. To find another origin of the difference in lipophilicity decrements between alkyl and aryl glucuronides, complete CLOGP calculations were performed for the aglycones and for the glucuronides. The CLOGP values correlate very well with experimental log *P* values of aglycones (*Eqn. 1* and *Fig. 2*) and glucuronides

(*Eqn. 5* and *Fig. 3*). However, the intercept of *Eqn. 2* (+ 0.87) is significantly different from zero and quite large. This fact suggests a systematic error in the CLOGP calculations of glucuronides which can be attributed to the glucuronyl moiety, since the CLOGP value of glucuronic acid (−3.37) is 0.82 log units more polar than the experimental value (−2.55).

$$\log P_{exp}(\text{aglycones}) = 0.92\ (\pm 0.07) \cdot \text{CLOGP} + 0.19\ (\pm 0.20) \quad (1)$$

$$n = 14;\ r^2 = 0.986;\ s = 0.19;\ F = 831$$

$$\log P_{exp}(\text{glucuronides}) = 1.03\ (\pm 0.10) \cdot \text{CLOGP} + 0.87\ (\pm 0.13) \quad (2)$$

$$n = 18;\ r^2 = 0.967;\ s = 0.26;\ F = 464$$

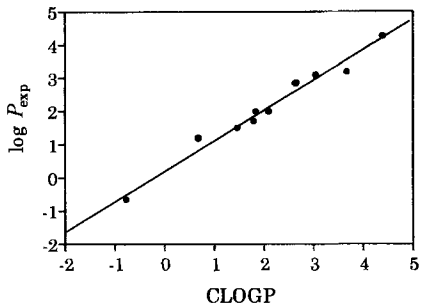

Fig. 2. *Correlation between the experimental log* P *and the calculated log* P (CLOGP) *of aglycones*

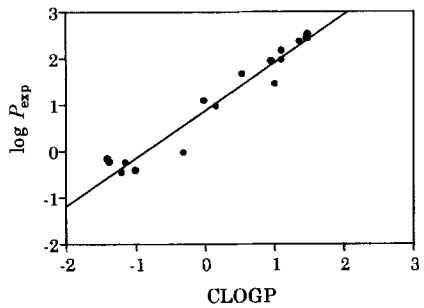

Fig. 3. *Correlation between the experimental log* P *and the calculated log* P (CLOGP) *of glucuronides*

The good correlation between the experimental and calculated CLOGP values of glucuronides suggests that the difference in lipophilicity decrements between aryl and alkyl glucuronides is mostly or only due to their 2D molecular topology. A detailed analysis of the CLOGP results reveals that only two corrections factors encode this difference, namely the proximity of two polar groups through one C-atom (+ 1.165 for alkyl glucuronides and + 0.778 for aryl glucuronides) and through a C,C bond (+ 0.848 for alkyl glucuronides and + 0.690 for aryl glucuronides).

In other words, through-bond proximity effects are responsible for a higher polarity of aryl glucuronides of 0.55 log *P* units relative to alkyl glucuronides. This effect of the proximity of two polar groups (0.55) accounts for most of the difference in experimental values (0.7–0.8 log *P* units) between alkyl and aryl glucuronides. Presumably conformational effects also play a role in modulating the lipophilicity of the glucuronides investigated here, but these effects appear modest and difficult to evaluate. Furthermore, no systematic trend emerged from virtual log *P* calculations due to the complex conformational behavior of these conjugates.

Interestingly, the difference in experimental lipophilicity between the aryl glucuronide **14g-ether** and the acyl glucuronides **14g-esters** of diflunisal is well-reproduced both by CLOGP calculations and by their *virtual* log *P* values. The higher lipophilicity of diflunisal acyl glucuronides (2.43 ± 0.06) compared to the phenolic glucuronide (1.66) can be attributed both to intrinsic differences (different atomic fragments) and to the presence in the acyl glucuronides only of a strong H-bond between the phenolic group and the ester linkage (*Fig. 4*).

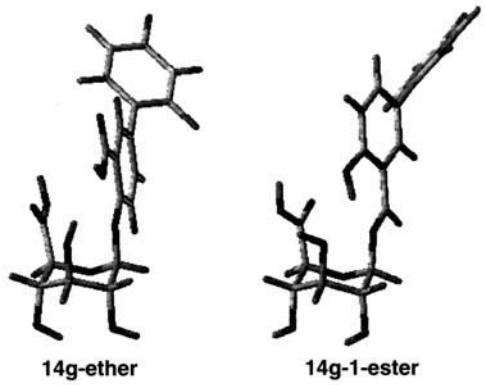

Fig. 4. *Most stable conformers of diflunisal ether β-D-glucuronide* (**14g-**ether) *and diflunisal ester β-D-glucuronide* **14g-***1-ester*

We also note that in contrast to the CLOGP algorithm, *Rekker*'s fragmental method [44] cannot be applied to glucuronides, its proximity corrections being too crude. Moreover, due to some limitations in their atomic fragments, neither the *Broto et al.* [42] nor the *Ghose* parameters [43] can reproduce the proximity effects in the glucuronyl moiety.

3. Conclusion. – Considering only the neutral forms, glucuronidation is shown here to decrease the lipophilicity of phenols (by -2.06 log P units) and carboxylic acids (by -1.80 log P units for diflunisal) more efficiently than that of alcohols (by -1.30 log P units). Differences in through-bond proximity effects as parametrized in the CLOGP algorithm seem to account for much of this difference. The influence of conformational factors, which is considered significant in the lipophilicity of morphine-*O*-glucuronides, appears modest and unassessable for the glucuronides investigated here.

The results presented in this study appear of interest in a pharmacokinetic perspective. Given their pK_a value of *ca.* 3, glucuronides exist mainly as anions in physiological fluids. The log P of carboxylic acids is known to decrease by *ca.* 3 units upon deprotonation, indicating that the log P of anionic glucuronides is too low to be measurable. In the body, both the anion and the neutral form of glucuronides may be involved in distribution and excretion. Our results imply that *in vivo* glucuronidation could facilitate the excretion of phenols more than that of alcohols. For carboxylic acids, glucuronidation also produces an important decrease in log P values, but, in contrast to phenols and alcohols, this is not done with an additional ionizable group being introduced, and thus the overall facilitation of excretion may be less important.

Experimental Part

Chemicals and Investigated Solutes. Anal. grade octanol, glycine, morpholinoethanesulfonic acid (MES), and morpholinopropanesulfonic acid (MPS) were obtained from *Fluka Chemie* (Buchs, CH). The following aglycones were investigated: methanol (**1a**; *Romil*, Cambridge, GB); (−)-(1*R*,2*S*,5*R*)-menthol (**2a**; *Siegfried*, Zofingen, CH); (+)-(1*S*,2*S*,5*R*)-neomenthol (**3a**), chloramphenicol (**4a**), phenol (**5a**), *p*-nitrophenol (**6a**), 4-methylumbelliferone (**9a**), phenolphthalein (**11a**), (dimethyl)(phenyl)methanol (**13a**) (*Fluka*); 6-bromo-2-naphthol (**8a**; *Aldrich*, Steinheim, D); and diflunisal (**14a**; *Sigma*, St. Louis, MO, USA). These compounds were of the best available purity

(usually > 99%) and were used without further purification. 3-Hydroxycoumarin (**10a**) was not commercially available, and its lipophilicity could not be measured but only calculated.

The following glucuronides were obtained from commercial sources: D-glucuronic acid (*Aldrich*), methyl β-D-glucuronide (**1g**), menthyl β-D-glucuronide (**2g**), chloramphenicol β-D-glucuronide (**4g**), phenyl β-D-glucuronide (**5g**), 1-naphthyl β-D-glucuronide (**7g**), 6-bromo-2-naphthyl β-D-glucuronide (**8g**), phenolphthalein 4′-*O*-β-D-glucuronide (**11g**) (*Sigma*); *p*-nitrophenyl β-D-glucuronide (**6g**) and 4-methylumbelliferyl β-D-glucuronide (**9g**) (*Fluka Chemie*). Four glucuronides were kind gifts from Prof. *John Caldwell* (St. Mary's Hospital Medical School, London): neomenthyl β-D-glucuronide (**3g**), 3-coumarinyl β-D-glucuronide (**10g**), 4′-benzophenonyl β-D-glucuronide (**12g**) and (dimethyl)(phenyl)methyl β-D-glucuronide (**13g**).

The purity of these glucuronides was examined by RP-HPLC using a *Kontron* chromatograph (pump *420*, autosampler *460*, data system *450*; *Kontron Instruments AG*, 8010 Zürich, CH) with UV detection (UV detector *430*, *Kontron*) and refractive index detection (*ERC-7512*, *Erma Cr. Inc.*, Tokyo, Japan). The column was a *C-18 Supelcosil LC-ABZ* (15 cm × 4.6 mm ID, 5 μm; *Supelco Inc.*, Bellefonte, PA, USA), and the eluents were MeOH/glycine 10^{-2} M pH 2.0 (20:80 to 80:20 (*v*/*v*)) with a flow rate of 1 ml/min [45]. It was found that the glucuronides from commercial sources had a purity of 98% or better, while the others had a purity of *ca.* 95%. Their lipophilicity being measured by CPC (see below), they could be used without further purification.

The glucuronides of diflunisal, diflunisal ether β-D-glucuronide (**14g**-ether) and diflunisal ester β-D-glucuronide **14g**-1-ester, were isolated from the urine of humans given the drug, and purified by RP-HPLC [46]. The isomers of the ester glucuronides, diflunisal ester 2-D-glucuronide **14g**-2-ester, diflunisal ester 3-D-glucuronide **14g**-3-ester, and diflunisal ester 4-D-glucuronide **14g**-4-ester, were formed by pH-dependent acyl migration of **14g**-1-ester prior to purification by RP-HPLC [46].

pK_a *Determinations*. The pK_a values were measured by potentiometry at 25° using degassed H_2O and were performed in triplicate. A *PCA 101* titrator (*Sirius Analytical Instruments Ltd.*, Forest Row, East Sussex, GB) was used. Samples ($5 \cdot 10^{-4}$–$4 \cdot 10^{-3}$ M) were dissolved in 0.1M KCl and titrated with 0.5N KOH (initial pH 2.5, final pH 10.0) at 25°. The electrode was standardized regularly according to the manufacturer's instructions. For control purposes, some titrations were also performed with a *Dosimat 665* and a *Titroprocesseur 670* (*Metrohm*, Herisau, CH), initial concentrations $1 \cdot 10^{-3}$–$5 \cdot 10^{-3}$ M dissolved in 0.1M KCl, titrating soln. 10^{-2} N NaOH or 10^{-2} N HCl. In this method, the pK_a values were calculated by a non-logarithmic linearization of the titration curve with correction for dilution [47][48]. Good agreement between the two methods was found.

Determination of Distribution Coefficients Using Centrifugal Partition Chromatography (CPC). Distribution coefficients in octanol/buffer were measured using an *Ito* multilayer coil separator-extractor (*P. C. Inc.*, Potomac, MD, USA), or a horizontal flow-through centrifugal partition chromatograph with a coil planet type centrifuge (*Pharma Tech Research Corp.*, Baltimore, MD, USA). The CPC technique employs a liquid-liquid partition system with the aid of centrifugal and *Archimedean* hydrodynamic forces, allowing a maximal retention volume of the stationary phase. It has been shown that it can circumvent problems inherent in the traditional shake-flask method such as impurities, instability of solutes, and low precision due to an unfavorable volume ratio of the org. and aq. phases [49]. The detailed exper. procedures and equations for the calculation of distribution coefficients can be found in [49][50].

The buffers and non-retained compounds were: pH 2: 10^{-2} M glycine or 10^{-2} M HCl, $CoCl_2$; pH 3: 10^{-2} M glycine, $CoCl_2$; pH 4, 5, and 6: 10^{-2} M MES, $NaNO_3$; pH 7.4: 10^{-2} M MPS, $K_2Cr_2O_7$. Biphenyl was used as the non-retained compound, when octanol was the eluent.

The log *D* values so obtained allowed the log *P* values of neutral forms to be calculated using *Eqn. 3* for acids and *Eqn. 4* for diacids:

$$\log P = \log D + \log(1 + 10^{pH - pK_a}) \quad (3)$$

$$\log P = \log D + \log(1 + 10^{pH - pK_{a1}} + 10^{2pH - pK_{a1} - pK_{a2}}) \quad (4)$$

$$\text{with } pK_{a1} < pK_{a2}$$

To ascertain that no artefact arose in CPC measurements of aromatic glucuronides, the log *D* of the 6-bromo-2-naphthyl β-D-glucuronide (**8g**) was measured by the micro-shake flask technique. The log *D* value of 1.53 ± 0.05 at pH 3.00 gave a log *P* of 1.87 ± 0.05 using *Eqn. 3*. This value is the same as that obtained by CPC (1.94 ± 0.02; see *Table*).

For a few highly lipophilic solutes, log *P* values were also measured by two-phase titration (*Sirius PCA 101* titrator), mainly to examine the partitioning of ionic species. The conditions were as described above for pK_a determinations, except for an ionic strength of 0.15 and an Ar atmosphere. The octanol/H_2O volume ratios were chosen according to the expected lipophilicity (0.5:15, 1:15, 1:4, or 1:1).

Conformational Studies Using Quenched Molecular Dynamics (QMD). The conformational behavior of each glucuronide was explored using a simplified conformational search strategy [39][51] able to describe efficiently the main valleys of a conformational space. Various starting geometries (4–6) were used and energy-optimized using the *Tripos* force field [52] with *Gasteiger-Marsili* formal atomic charges [53] in order to remove initial high-energy interactions. High-temp. molecular dynamics (MD) calculations were carried out at 2000 K. Each simulation was run for 100 ps with steps of 1.0 fs. The frame data were stored every 0.05 ps, giving 2000 frames. The starting velocities were calculated from a *Boltzmann* distribution. Finally, 10% of all conformers were randomly selected and saved in a database ultimately containing *ca.* 200 conformers.

All conformers in the database were then subjected to energy-minimization using the same force field as for the MD calculations. The *Powell* minimization method was applied with the gradient value of 0.001 to test for convergence. The maximum number of iterations was set at 3000. The energy-minimized conformers were then classified according to increasing energy.

The conformational similarity of the 200 energy-minimized conformers was investigated by comparing all pairs of conformers. The two criteria of comparison were the force-field energy and the RMS distance difference calculated by the option MATCH of SYBYL over all heavy atoms and polar H-atoms. An *ad hoc* Fortran program then calculated the mean and standard deviations of the RMS values. Two conformers were considered identical when their energy difference was ≤ 3 kcal/mol and their RMS distance difference less than or equal to the RMS mean minus the standard deviation. When this was the case, one of the two conformers was eliminated from the database, and it was always the one of higher energy.

All calculations were run on *Silicon Graphics Personal Iris 4D-35*, *Indigo R4000* or *Indy R4400* workstations. SYBYL 6.2 molecular modeling package (*Tripos Associates*, St. Louis, MO, USA) and MOPAC (*QCPE*, No. 445) were used.

Calculation of the Molecular Lipophilicity Potential (MLP). The Solvent-Accessible Surface Area (SASA) [54] of the conformers generated by QMD was utilized as the space for integrating the MLP back to log P_{oct} values using *Eqn. 5* [40]:

$$\log P_{oct} = 2.86 \cdot 10^{-3} (\pm 0.24 \cdot 10^{-3}) \Sigma MLP^{+} + 1.52 \cdot 10^{-3} (\pm 0.22 \cdot 10^{-3}) \Sigma MLP^{-} - 0.10 (\pm 0.23) \quad (5)$$

$$n = 114;\ r^2 = 0.94;\ s = 0.37;\ F = 926$$

where ΣMLP^{+} and ΣMLP^{-} represent the hydrophobic and polar parts of the molecule, respectively. The MLP calculations were performed with the CLIP 1.0 software [55].

The most lipophilic and hydrophilic conformers were retained and the difference between their *virtual* log *P* values (virtual log *P* being the log *P* calculated for a given conformer [54]) was considered as the lipophilicity range accessible to a given solute in the neutral state. The lipophilicity range encompasses the ensemble of all virtual log *P* values of a solute, whereas the experimental log *P* is the weighted average of an unknown number of virtual log *P* values of the molecule [54].

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