CCXXXIX. STUDIES IN DETOXICATION

II. (a) THE CONJUGATION OF ISOMERIC 3-MENTHANOLS WITH GLUCURONIC ACID AND THE ASYMMETRIC CONJUGATION OF *dl*-MENTHOL AND *dl*-isoMENTHOL IN THE RABBIT. (b) *d*-isoMENTHYLGLUCURONIDE, A NEW CONJUGATED GLUCURONIC ACID

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WHEN terpenes (or their derivatives) are administered to animals, a considerable proportion is excreted as hydroxy derivatives conjugated with glucuronic acid; for example, 80% of borneol is conjugated in this way in man [Pryde & Williams, 1936]. It was from the conjugated glucuronides formed after the feeding of camphor to dogs that *d*-glucurone was first isolated [Schmiedeberg & Meyer, 1879; cf. Jaffe, 1878]. Conjugation with glucuronic acid does not, however, account for all the terpene administered and it is probable that some portion undergoes complete oxidation. This work deals only with the conjugation.

The main objects of the present investigation were to find out whether the optical (enantiomorphic) and geometrical isomerism of the menthols (3-menthanols) influenced their conjugation with glucuronic acid in the body and whether the feeding of a *dl*-menthol resulted in the excretion of a conjugated glucuronide containing more of one antipode than the other.

The conjugation of menthol and isomenthol

It has already been shown by Quick [1932] and by Williams [1938] that conjugation can be influenced by position isomerism, i.e. *o*-, *m*- and *p*-substitution. The terpene alcohols, the menthanols, exhibit three types of isomerism, namely structural (e.g. 2-menthanol and 3-menthanol), geometrical (e.g. menthol and *isomenthol*) and optical isomerism (e.g. *d*- and *l*-menthol). The present investigation deals with the 3-menthanols alone. Four¹ of these are theoretically possible, each occurring in *d*-, *l*- and *dl*-forms [Simonsen, 1931]. Only two have been available and these are menthol (I and II) and *isomenthol* (III). The forms used were *l*- and *dl*-menthol and *d*- and *dl*-*isomenthol*.

¹ Menthol, *neomenthol*, *isomenthol* and *neoisomenthol*.

The mean percentages of these substances conjugated with glucuronic acid in the rabbit under standard conditions (dose 1 g./kg.) are summarized in Table I. The calculated values for *d*-menthol and *l*-isomenthol are also shown.

Table I. *The percentage conjugation of menthol and isomenthol with glucuronic acid*

Menthol	% conjugated	isoMenthol	% conjugated
<i>l</i> -	48	<i>l</i> -	45 (calc.)
<i>dl</i> -	59	<i>dl</i> -	55
<i>d</i> -	70 (calc.)	<i>d</i> -	65

Table I shows that there is only a slight difference between the conjugation values of menthol and isomenthol provided that similar forms are compared, but there is a pronounced difference between those of their optical antipodes. The conjugation of either of the *d*-antipodes is nearly 50 % greater than that of either of the *l*-antipodes. The conclusion is therefore drawn that the conjugation of these two menthanols with glucuronic acid in the rabbit is little influenced by their geometrical isomerism but considerably affected by their optical isomerism.¹ Further, the menthanols possessing the *d*-configuration are more easily conjugated and therefore presumably more resistant to biological oxidation than those of the *l*-configuration.

The asymmetric conjugation of dl-menthol and dl-isomenthol

Since one optical isomeride is conjugated to a greater extent than its enantiomorph, it follows that on feeding an optically inactive *dl*-mixture, the excreted glucuronide should contain more of one isomeride than of the other. It has already been shown qualitatively that after feeding *dl*-borneol, *dl*-isoborneol [Hämäläinen, 1909], *dl*-menthol [Jacobsohn & da Cruz, 1929; 1930] or *dl*-*p*-sec-butylphenol [Fromageot, 1929], these compounds are excreted in conjugated forms which contain unequal amounts of the original *d*- and *l*-compounds. Administration of *dl*-camphor also results in the excretion of an optically active campherol [Meyer, 1908], but in this case the result may be complicated by the fact that at least two campherols (3- and 5-hydroxycamphors) are formed after the feeding of camphor to dogs [Asahina & Ishidate, 1928]. It has also been shown [Pryde & Williams, 1934] that when commercial *d*-borneol is fed to dogs, the borneol excreted in conjugation with glucuronic acid contains a greater proportion of the *d*-isomeride than that fed. None of this work was quantitative, since no estimations were made of the extent of conjugation of either of the optical antipodes or of the *dl*-compound itself. Further, conjugated glucuronides such as *d*-menthyl- β -*d*-glucuronide and *l*-menthyl- β -*d*-glucuronide, are not optical antipodes and therefore fractional crystallization is possible during the isolation of the mixed glucuronides after the feeding of a *dl*-compound. Hence the amount of each isomeride in the compound isolated may depend to some extent on a difference in solubility between the two glucuronides. Table I shows that *d*-menthol is conjugated to greater extent than *l*-menthol; if *dl*-menthol behaves in the body as a simple mixture of equal proportions of *d*- and *l*-menthol, then the excreted glucuronide after feeding *dl*-menthol should contain more

¹ The term "optical isomerism" is here used in the limited sense of "enantiomorphism". All the geometrical isomerides of 3-menthanol possess, in addition to a cyclic structure, three asymmetric carbon atoms and are optically active. They are therefore, broadly speaking, optical isomerides, but they are not all enantiomorphs.

d-menthol than *l*-. From the figures in Table I it can be calculated that the menthol recovered after the administration of *dl*-menthol should have $[\alpha]_D$ about $+10^\circ$ in alcohol (taking menthol as $[\alpha]_D \pm 50^\circ$ in alcohol), i.e. about 60% *d*- and 40% *l*-. In duplicate experiments values of $+14.4^\circ$ and $+12.8^\circ$ (in alcohol) were obtained and these figures correspond to *d*- 64%, *l*- 36% and *d*- 63%, *l*- 37% respectively. A similar argument can be applied to *dl*-isomenthol, the calculated value of the rotation being about $+5^\circ$ (in alcohol) which corresponds to 59% *d*- and 41% *l*- (isomenthol has $[\alpha]_D \pm 26^\circ$ in alcohol). The figures found experimentally with isomenthol were $+6.3^\circ$ (i.e. 62% *d*- and 38% *l*-) and $+5.8^\circ$ (i.e. 61% *d*- and 39% *l*-). The optical measurements are therefore in fair agreement with values calculated from the chemical analyses quoted in Table I (see also Table III).

d-isoMenthyl- β -*d*-glucuronide

During the progress of this work a new conjugated glucuronic acid has been isolated from the urine of rabbits following the administration of *d*-isomenthol. The probable structure of this product (*d*-isomenthylglucuronide) is shown in the accompanying formula (IV). A description of the acid and some of its derivatives is given in the experimental section.

It has also been found that *dl*-menthol and *dl*-isomenthol give good yields of their respective glucuronides when fed to rabbits. This suggests their possible use as profitable biosynthetic sources of glucuronic acid [cf. borneol, Quick, 1927].

EXPERIMENTAL

Female rabbits, fed on a diet of 100 g. cabbage and 50 g. bran a day, were used under conditions similar to those already described [Williams, 1938]. *dl*-isoMenthol,¹ *l*- and *dl*-menthols were fed in warm water emulsions and *d*-isomenthol in aqueous suspension by stomach tube. The urine was collected for 2 days and analysed for conjugated glucuronic acid by ether extraction in a small continuous extractor (extracting 10 ml. urine in 3 hr.). The ethereal extract was evaporated and the residue hydrolysed by acid and then neutralized. The free glucuronic acid was estimated with Benedict reagent by the Quick method [1925]. The Benedict reagent was standardized against analytically pure *d*-isomenthylglucuronide, $C_{16}H_{28}O_7$, H_2O , and glucose. The ratio of the amounts of glucuronic acid and glucose required to reduce a given volume of Benedict reagent was 1.098 (calc. 1.077). The experimental results for the percentage conjugations of the 3-menthanols are given in Table II, whilst the average figures are given in Table I.

¹ Rotations: $[\alpha]_D$ in alcohol: *l*-menthol (B.D.H.), -50.6° ($c=1.4$); *d*-isomenthol (Howard, Ilford), $+25.2^\circ$ ($c=1.0$); *dl*-menthol (Howard, Ilford), 0° ; *dl*-isomenthol (Howard, Ilford), 0° .

Table II. *The percentage conjugations of menthol and isomenthol with glucuronic acid*

Substance	Rabbit no.	Wt. of rabbit (kg.) and dose (g.)	Urine vol. ml.	Glucuronic acid g.	Menthol conjugated g.	Menthol conjugated %
<i>l</i> -Menthol*	2	2	224	0.996	0.801	40
	5	2.4	357	1.393	1.120	47
	6	2.4	358	1.582	1.273	53
	2	2	265	1.345	1.081	54
<i>dl</i> -Menthol	1	2	248	1.514	1.217	61
	2	2	199	1.458	1.173	59
	3	2	254	1.444	1.161	58
<i>d</i> -isoMenthol	1	2	224	1.743	1.402	70
	2	2	273	1.567	1.260	63
	1	2	307	1.511	1.214	61
<i>dl</i> -isoMenthol	2	2	314	1.484	1.194	60
	1	2	216	1.364	1.097	55
	1	2	190	1.226	0.986	49

* In two experiments with *l*-menthol Quick [1924] found values of 42 and 47.5%, the dose being 2 g.

Experiments on asymmetric conjugation

(a) *dl*-Menthol. A rabbit was given 3 g. of *dl*-menthol in warm water by stomach tube and the urine collected for one or two days (see Table III). The urine was made slightly alkaline with NH_4OH and the precipitate of phosphates removed. To the filtrate solid $(\text{NH}_4)_2\text{SO}_4$ (50 g./100 ml.) was added and the whole boiled and filtered. The filtrate was kept in the refrigerator overnight. The crystalline precipitate of ammonium menthylglucuronate was separated, dried *in vacuo* and weighed. This salt was then hydrolysed by boiling under reflux for 1 hr. with 10% H_2SO_4 . The hydrolysed solution was steam-distilled and the distillate cooled in ice. The solidified menthol was collected from the distillate, carefully dried with filter paper and weighed. The m.p. of the recovered menthol was 25–28°. Its specific rotation was measured in absolute alcohol (see Table III).

Table III. *The recovery of menthol and isomenthol from urine after the feeding of their dl-forms*

Rabbit no.	Substance fed	Dose g.	Urine vol. ml.	Menthol recoverable g.	Menthol recovered in NH_4 salt g.	Menthol from steam distillation g.	$[\alpha]_D$	$[\alpha]_D$ calc.
6	<i>dl</i> -Menthol	3	202*	1.77 (59%)	1.6	1.5	+14.4°	+ 10°
3		3	300†	1.77 (59%)	1.5	1.3	+12.8°	
4	<i>dl</i> -isoMenthol	2.5	222*	1.4 (55%)	1.2	1.0	+6.3°	+ 5°
5		3	197*	1.7 (55%)	1.7	1.6	+5.8°	

* In 1 day.

† In 2 days.

Rotations in absolute alcohol.

(b) *dl*-isoMenthol. The procedure used was the same as for *dl*-menthol except that the filtration after adding $(\text{NH}_4)_2\text{SO}_4$ was omitted. This omission was necessary since ammonium isomenthylglucuronate would not completely re-dissolve in the boiling urine after the addition of $(\text{NH}_4)_2\text{SO}_4$. The recovered isomenthol had m.p. 40–42° (see Table III).

d-isoMenthyl- β -D-glucuronide and its derivatives

d-isoMenthylglucuronide can be isolated from urine directly as the free acid or, after precipitation with $(\text{NH}_4)_2\text{SO}_4$, as the ammonium salt.

(a) *Isolation as free acid.* The isolation of the free acid is an exceedingly simple procedure, since the compound is precipitated from the urine when the latter is acidified. Rabbits were given 2–3 g. of *d*-isomenthol with water by stomach tube. The excretion of the glucuronide was practically complete within 24 hr. The urine was collected after one or two days and, after filtering through cotton wool, was made strongly acid with 20% (wt./wt.) H_2SO_4 (25 ml. acid to 100 ml. urine). Warming to 40° accelerates the separation. The glucuronide began to separate immediately and the process was complete after several hours in the refrigerator. The compound was then filtered under suction, washed with water and dried. In most cases the crude glucuronide had a pinkish colour due to urinary pigments, but in some cases an almost pure white product was obtained. One recrystallization from hot water (charcoal) gave a white crystalline product, M.P. ca. 120° . The yield was 0.7 g. per g. of *d*-isomenthol fed (see Table IV).

(b) *Isolation as ammonium salt.* The urine was made slightly alkaline with ammonia, boiled and the precipitate of phosphates removed by filtration. To the filtrate solid $(\text{NH}_4)_2\text{SO}_4$ was added (30 g./100 ml.) and the solution boiled. Immediate precipitation of the ammonium *d*-isomenthylglucuronate took place and the process was completed in the refrigerator. The crude salt was then separated and dried. The yield was 1.2 g. per g. of *d*-isomenthol fed. The recovery of material was better than by the first method (see Table IV).

Table IV. *The recovery of d-isomenthylglucuronide from urine*

Rabbit no.	Dose g.	Yield as free acid g.	Rabbit no.	Dose g.	Yield of NH_4 salt g.
1	2	1.6	5	2.5	3.0
1	2	1.4	6	2.5	2.8
2	2	1.0	7	2.5	3.2
4	2.5	1.75			
6	2.5	1.8			

d-isoMenthyl- β -D-glucuronide. The crude glucuronide after two recrystallizations from a large volume of hot water was obtained pure. It forms long fine matted needles, M.P. 126° (corr.); it is very sparingly soluble in cold water, but easily soluble in hot water, ethyl alcohol, methanol and ether. It crystallizes from water with $1\text{H}_2\text{O}$ and has $[\alpha]_D^{16} - 43.2^\circ$ ($c = 1.2$ in abs. alcohol). (Titration: 114.75 mg. required 16.48 ml. of 0.0198 *N* NaOH; calc. 16.55 ml. Found: C, 54.75; H, 8.6; H_2O , 5.0%. $\text{C}_{16}\text{H}_{28}\text{O}_7$, H_2O requires C, 54.8; H, 8.6; H_2O , 5.1%.)

Ammonium d-isomenthyl- β -D-glucuronate. The crude ammonium salt was purified by dissolving in hot water, filtering and precipitating with $(\text{NH}_4)_2\text{SO}_4$. Three such treatments gave an analytically pure salt as glistening plates. The salt was anhydrous and had $[\alpha]_D^{18} - 41.1^\circ$ ($c = 1.3$ in water). (Found: N, 4.02%. $\text{C}_{16}\text{H}_{31}\text{O}_7\text{N}$ requires N, 4.01%.)

Cadmium d-isomenthyl- β -D-glucuronate. Aqueous solutions of the ammonium salt gave amorphous precipitates with soluble Cu, Fe, Zn, Ag and Co salts. On adding aqueous CdCl_2 to a solution of the above ammonium salt and slightly warming the mixed solutions, an insoluble crystalline cadmium salt separated as shining platelets. (Found: Cd, 14.7%. $(\text{C}_{16}\text{H}_{27}\text{O}_7)_2\text{Cd}$ requires Cd, 14.5%.)

Hydrated cadmium salts of *l*-menthylglucuronide have been prepared by Fromm & Clemens [1901], but the present compound was anhydrous and lost no weight at 110°.

The recovery of conjugated glucuronic acid after the feeding of dl-menthol and dl-isomenthol

By using the $(\text{NH}_4)_2\text{SO}_4$ precipitation method already described, good yields of crude ammonium menthyl- and isomenthyl-glucuronates can be obtained from the urines after feeding the *dl*-compounds. The yields were 1.1 to 1.4 g. per g. of the *dl*-menthanols fed (see Table V).

Table V. *Yields of glucuronides after dl-menthol and dl-isomenthol*

Exp.	Substance	Dose g.	Yield of crude NH_4 salt g.
1	<i>dl</i> -Menthol	3	4.1
2	"	3	3.3
3	"	2	2.5
4	<i>dl-iso</i> Menthol	2.5	3.0
5	"	3	4.4

These ammonium salts are of course mixtures containing more of the glucuronides of the *d*- than of the *l*-isomerides.

The glucuronide after feeding dl-menthol. Some of the crude ammonium salt obtained after feeding *dl*-menthol was further purified and converted into the free acid by adding the requisite amount of dilute HCl. The free acid was recrystallized from hot water, crystallization being accelerated by adding a little dilute HCl to the cooled solution. One purified specimen had m.p. 103–104° and $[\alpha]_D^{19} - 41.8^\circ$ ($c=2$ in alcohol) (pure *l*-menthylglucuronide has $[\alpha]_D - 105^\circ$ in alcohol [Fischer, 1910]). Like the pure *l*-derivative this mixture crystallizes with 1.5 H_2O . (Titration: 108.6 mg. required 15.16 ml. of 0.01994 *N* NaOH; calc. for $\text{C}_{16}\text{H}_{28}\text{O}_7$, 1.5 H_2O , 15.17 ml.)

The glucuronide after dl-isomenthol. A specimen of the crude ammonium salt recovered after the feeding of *dl-isomenthol* was subjected to two further recrystallizations from hot half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The purified material had $[\alpha]_D^{18} - 54.4^\circ$ ($c=1.6$ in water); pure ammonium *d-isomenthyl*-glucuronate has $[\alpha]_D - 41^\circ$ (in water).

It must be pointed out, however, that *d*- and *l*-menthyl- (or *-isomenthyl*-) glucuronides are not optical antipodes and therefore the purification of the mixed free acids or ammonium salts will result in some measure of fractional crystallization. The measured specific rotations will therefore be somewhat variable. Experience gained in this work shows that the *l*-compounds are more soluble than the *d*-menthylglucuronides. The separation of the *l*- and *d*-menthylglucuronides is being further investigated.

SUMMARY

1. The extents of conjugation of the geometrical isomerides, menthol and isomenthol, with glucuronic acid in the rabbit are almost identical, provided that similar optical isomerides are compared. The optical isomerides (enantiomorphs), *d*- and *l*-menthol and *d*- and *l-isomenthol* show pronounced differences in conjugation, the *d*-compounds conjugating to nearly a 50% greater extent than the *l*-.

2. Asymmetric conjugation with glucuronic acid takes place when *dl*-menthol and *dl*-isomenthol are fed to rabbits. The menthol and isomenthol recovered from the urine have positive rotations which agree with the values calculated from the percentage conjugations of the separate *d*- and *l*-isomerides.

3. The preparation and properties of *d*-isomenthyl- β -*d*-glucuronide and of two of its derivatives are described for the first time.

4. It is suggested that *dl*-menthol and *dl*-isomenthol can be used as profitable sources of biosynthetic glucuronic acid.

The *dl*-menthol, *d*- and *dl*-isomenthol used in this work were supplied by Messrs Howard and Sons, Ltd., Ilford.

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