CLXXXVI. STUDIES IN DETOXICATION

III. THE USE OF THE GLUCURONIC ACID DETOXICATION MECHANISM OF THE RABBIT FOR THE RESOLUTION OF *dl*-MENTHOL

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RESOLUTION of racemic substances as a result of selective assimilation by living organisms is originally due to Pasteur [1858, 1860] who showed that *Penicillium glaucum* destroyed the *d*-tartrate in a solution of ammonium racemate leaving the *l*-isomeride. Examples of the action of micro-organisms in this manner are numerous [see Cohen, 1924].

The mammalian organism also deals with optical antipodes selectively and this fact has been utilized in a few cases to produce a partial separation of the antipodes, when they are excreted in the urine following their introduction into the body. Thus, when sodium *dl*-phenyl- γ -hydroxybutyrate is given to dogs, more *l*-isomeride than *d*- is found in the urine [Thierfelder & Schempp, 1917]; sodium *dl*-malate injected subcutaneously into rabbits gives *d*-malate in the urine [Tomita, 1921] and intravenous injection of sodium *r*-mandelate into cats results in the preferential excretion of the *l*-form at first, the *d*-form predominating later [Garry & Smith, 1938]. In the case of terpenes, it has been shown that the *dl*-alcohols of this series are detoxicated in the animal by conjugation with glucuronic acid and the glucuronide so formed contains more of one isomeride than of its enantiomorph [cf. Williams, 1938]. The object of the present investigation is to show that advantage can be taken of this asymmetric conjugation for the resolution of *dl*-menthol and possibly other terpenes.

The resolution of dl-menthol

Within recent years, *dl*-menthol has been resolved very successfully by Read and his co-workers [Read & Grubb, 1931; 1932; Clark & Read, 1934], whose methods depend upon fractional crystallization of the esters of *dl*-menthol with optically active acids. By Read's methods, *d*-menthol, previously regarded as a chemical curiosity, was easily obtained in excellent yields, together with *l*-menthol, from synthetic *dl*-menthol. More recently Barrow & Atkinson [1939] have resolved *dl*-menthol through its ester with (+)-tartranilic acid, although only *l*-menthol is obtained by this method. In the present paper the resolution of *dl*-menthol has been accomplished by a partially biochemical method which depends on glucuronide formation in the rabbit. Neuberg *et al.* [1928-9] have shown that *dl*-menthol can be resolved to give optically pure *l*-menthol (but not *d*-) through the glucoside; if *dl*-menthol is condensed with acetobromoglucose in the presence of quinoline, *l*-menthol tends to form an α -, whilst *d*-menthol tends

to form a β -glucoside, and these can be separated. If, however, *dl*-menthol is condensed in the presence of silver carbonate only β -glucosides are obtained and little separation of these is possible. Neuberg *et al.* also showed that emulsin, acting on *dl*-menthyl- β -glucoside, hydrolyses the *d*-form quicker than the *l*-, but optically pure menthols could not be obtained in this manner. In the present work the *d*- and *l*-menthylglucuronides obtained from the rabbit are both β -glycosides and can be separated by fractional crystallization. Three different procedures, described below, were adopted. Two of these gave *d*-menthol in about 10% yield (calc. on *dl*-menthol fed); the third method gave no further separation than that already performed by the organism of the rabbit.

(a) Oral administration of *dl*-menthol to rabbits results in the excretion of a diastereoisomeric pair, namely *d*- and *l*-menthyl- β -*d*-glucuronide, the former being more abundant than the latter. These diastereoisomerides as ammonium salts showed sufficient difference in solubility to be separated by fractional crystallization from water and ammonium sulphate solutions. The free acids could not be separated with any success. In this manner, 10 g. of the mixed ammonium menthylglucuronates could be made to yield 1–1.5 g. of ammonium *d*-menthylglucuronate.

(b) When *dl*-menthol is fed to rabbits, the *l*-isomeride is oxidized to a greater extent than the *d*- [Williams, 1938]. It should, therefore, be possible to destroy most of the *l*-form by taking advantage of this selective oxidation. *dl*-Menthol was fed to rabbits and the excreted glucuronide (NH_4 salt had $[\alpha]_D$ ca. -28° , and contained 30–35% *l*-) was hydrolysed with dilute acid, the menthol formed being simultaneously steam-distilled. The main bulk of the menthol recovered had $[\alpha]_D$ ca. $+20^\circ$ in alcohol. During this hydrolysis it was observed that the earlier fractions of menthol had a higher dextrorotation than later ones (see Table III) indicating that the rate of hydrolysis of *d*-menthylglucuronide is greater than that of the *l*-form. The recovered menthol was now re-fed to rabbits and the excreted glucuronide ($[\alpha]_D$ ca. -10°) now contained 85–90% of the *d*-isomeride. One recrystallization of the ammonium salt of this glucuronide from water containing 5% ammonium sulphate gave mainly *d*-isomeride, 10 g. of the mixed salts yielding 5–7 g. of the *d*-salt.

(c) It was thought possible that if the urine excreted by rabbits fed with *dl*-menthol were collected periodically it might be found that preferential excretion of one of the diastereoisomerides took place. This, however, was not realized since all fractions obtained had the same rotation and contained the same proportion of *d*- to *l*-menthol (see Table II).

Optically pure *l*-menthylglucuronide was not obtained from the mixed glucuronide since the amount of it occurring in the latter is much smaller than that of the *d*-. In one experiment by the first method above, an ammonium salt showing $[\alpha]_D -84^\circ$ (i.e. 80% *l*-) was obtained.¹

It is interesting to note that all the known natural conjugated glucuronic acids are laevorotatory. *d*-Menthylglucuronide appears to be the first to be discovered with a dextrorotation, albeit a small one. There is no reason to believe that it has other than a β -glycosidic structure. Table I summarizes the rotations and melting points of the known glucuronides of *p*-menthan-3-ol.

¹ 12 g. of ammonium *l*-menthylglucuronate were isolated from rabbit urine after feeding 18 g. *l*-menthol. The purified salt (needles of monohydrate) showed $[\alpha]_D^{26} -103.4^\circ$ ($c=1.8$ in water). *l*-Menthylglucuronide forms crisp plates (monohydrate) with m.p. 94° and $[\alpha]_D^{26} -110.7^\circ$ ($c=2$ in alcohol).

Table I. *The glucuronides of p-menthan-3-ol*

Glucuronide	M.P.	$[\alpha]_D$ in alcohol	Author
<i>l</i> - α -Menthyl (synthetic)	130°	+ 52°	Bergmann & Wolff [1923]
<i>l</i> - β -Menthyl	94°	- 110°	See footnote on p. 1520
<i>d</i> - β -Menthyl	120°	+ 6°	This paper
<i>d</i> - β - <i>iso</i> Menthyl	126°	- 43°	Williams [1938]

Isolation of d-menthol

This menthol was obtained by acid hydrolysis of *d*-menthylglucuronide. The yield was almost quantitative and it had M.P. 41° and $[\alpha]_D + 49.4^\circ$ in alcohol (Read & Grubb [1934], give M.P. 42–43° and $[\alpha]_D + 49.6^\circ$). Pure *d*-menthylglucuronide was obtained by acidification of solutions of the ammonium salt obtained from the resolutions. If *d*-menthol is prepared by hydrolysis of this ammonium salt, a specimen which is not optically pure ($[\alpha]_D + 46^\circ$) is obtained. This ammonium salt was found to contain a small amount of an unknown menthanolglucuronide, which was easily separated during the conversion of the NH_4 salt into the free acid (see experimental). This unknown glucuronide crystallizes in needles and its NH_4 salt forms plates. This crystal habit is typical of *d*-isomenthylglucuronide and its NH_4 salt [Williams, 1938] and of ammonium *dl*-isomenthylglucuronide. In the case of the *d*- and *l*-menthylglucuronides, the free acids form plates and the NH_4 salts needles. This unknown glucuronide may be an *isomenthyl* derivative, since Read & Grubb [1932] state that commercial *dl*-menthol often contains an appreciable amount of *dl*-isomenthol.

EXPERIMENTAL

Isolation of ammonium menthylglucuronate. Rabbits (2–2.5 kg.) on a diet of 100 g. cabbage and 50 g. bran a day were each given 3 g. *dl*-menthol ($[\alpha]_D 0^\circ$ in alcohol) by stomach tube in about 15 ml. water warm enough to melt the menthol (M.P. 34–37°). The urine was collected for about 30 hr. and after filtration through muslin, was made slightly alkaline with a few drops of ammonia (sp.gr. 0.880), boiled and filtered to remove precipitated phosphates. The filtrate was treated with 50 g. of ammonium sulphate per 100 ml. and brought to the boil with occasional shaking. The boiling urine was then filtered hot through a coarse fast filter paper to remove a precipitate of protein which appeared to be of a mucoid nature. The filtrate was now kept overnight in the refrigerator and ammonium menthylglucuronate crystallized in needles which filled the solution. The yield of crude dry salt was 1.2–1.4 g. per g. *dl*-menthol fed and a typical preparation had $[\alpha]_D - 28.3^\circ$ ($c=1$ in water).

The rate of excretion of menthylglucuronide. Rabbits were given 3 g. each of *dl*-menthol and the urine was collected periodically during 48 hr. The NH_4 salt was isolated from each fraction by the method indicated above. In order to ensure as complete a separation of the salt as possible, the time for crystallization was prolonged to 2 days at 0°. A control experiment to check the yield was performed, the urine being collected and worked up in one batch. The result of such an experiment using 6 rabbits is given in Table II. It can be seen from the table that the excretion of conjugated menthol is complete in about 30 hr. The bulk (75 %) of the material is excreted in the first 12 hr.

From the rotations of the various fractions (Table II) it can be seen that the proportion of *d*- to *l*-menthylglucuronide is constant and there is no evidence of preferential excretion of either one of them. Little significance can be attached

Table II. *The rate of excretion of menthylglucuronide after dl-menthol in rabbits*A. 6 rabbits given 3 g. *dl*-menthol each and the urine collected periodically.B. 6 rabbits given 3 g. *dl*-menthol each and the urine collected in one batch.

Time after feeding hr.	A		B	
	Yield dry NH ₄ salt g.	$[\alpha]_D$ in water	Yield dry NH ₄ salt g.	$[\alpha]_D$ in water
6	6.5	-26.6°	—	—
12	12.4	-25.8°	—	—
24	3.6	-27.1°	—	—
31	2.0	-16.5°	—	—
42	—	—	25	-28°
48	0.0	—	—	—
	Total 24.5			

to the low rotation of the last fraction since it is easy to lose a small amount of the more soluble *l*-menthylglucuronate. Owing to the high negative rotation of the latter ($[\alpha]_D - 103^\circ$), loss of a small amount will have a considerable effect on the rotation of the mixed salts.

Fractionation of the salt $[\alpha]_D - 28^\circ$. A typical resolution of this salt was carried out as follows: 10 g. of the salt were dissolved in 50 ml. water with warming; after an hour, 2.1 g. of a salt of rotation -16.6° were isolated. This material was now dissolved in 15 ml. of warm water, and on standing a small amount of material with $[\alpha]_D - 33^\circ$ separated. The latter was removed and a little solid ammonium sulphate was stirred into the solution; 0.7 g. of ammonium *d*-menthylglucuronate ($[\alpha]_D + 5^\circ$) separated. The filtrate from the fraction with $[\alpha]_D - 16.6^\circ$ yielded another 0.7 g. of the *d*-salt on further fractionation. Recrystallization from water of the salt with $[\alpha]_D + 5^\circ$ failed to alter its rotation. The more soluble fractions gave, on fractional precipitation with increasing concentrations of ammonium sulphate, a small amount of material with $[\alpha]_D - 84^\circ$ which, therefore, contained over 80 % *l*-isomeride (the rotations are in water).

*Isolation of ammonium d-menthylglucuronate after feeding
recovered menthol*

(a) *Hydrolysis of the salt* $[\alpha]_D - 28^\circ$. 10 g. of the salt obtained after feeding *dl*-menthol were dissolved in 100 ml. water and 15 ml. of 2*N* H₂SO₄ were added. The free menthylglucuronide was extracted from the aqueous solution with ether. The ether was removed and the residue taken up in 100 ml. of 0.4*N* H₂SO₄. The menthylglucuronic acid was hydrolysed by a steam-distillation of 2 hr. duration, the menthol formed being simultaneously collected in the distillate. The residual solution was usually worked up for the preparation of glucurone. In one experiment in which 92 g. of the mixed salt were hydrolysed, the menthol

Table III. *Recovery of menthol after hydrolysis of ammonium menthylglucuronate* $[\alpha]_D - 28^\circ$

Fraction	Yield g.	$[\alpha]_D$ in alcohol
1	1.6	+28°
2	1.7	+24°
3	25.0	+20°
4	3.0	-3°

was collected in four fractions. The rotations of these fractions are given in Table III.¹ The significance of these figures has already been pointed out.

(b) *Isolation of the glucuronide after feeding recovered menthol.* 30 g. of the recovered menthol (fractions of low rotation were not used) were fed to 10 rabbits each receiving 3 g. of the crude material. After 2 days the urine was worked up in the usual manner and 34 g. of crude ammonium salt were isolated with $[\alpha]_D - 9.7^\circ$ in water.

The fractionation of this salt was carried out as follows: the salt (17.6 g.) was dissolved in 180 ml. water with heating and the solution filtered. Ammonium sulphate (10 g.) was stirred into the solution and 12 g. of crude ammonium *d*-menthylglucuronate ($[\alpha]_D$ 0 to $+1^\circ$ in water) were isolated. Treatment of the filtrate with 20 g. ammonium sulphate gave a second fraction (4.1 g.) with $[\alpha]_D - 36^\circ$ in water. Recrystallization of the second fraction from water yielded a small amount of an ammonium salt which crystallized in plates and had $[\alpha]_D - 53.3^\circ$ in water. The latter is probably ammonium *dl*-isomenthylglucuronate, since the authentic salt has been prepared by the author during another investigation (unpublished) and found to have $[\alpha]_D$ ca. -54° .

Preparation of d-menthol-β-d-glucuronide. Ammonium *d*-menthylglucuronate (5 g.) as obtained above was dissolved in hot water (100 ml.) and the solution filtered. The filtrate was treated with twice the calculated amount of 2*N* H₂SO₄. On cooling the solution to 0°, almost pure *d*-menthylglucuronide separated in large lustrous plates (yield 80%). After two recrystallizations from water and drying over CaCl₂, it melted at 120–122° (uncorr.) and had $[\alpha]_D^{20} + 6.4^\circ$ (*c* = 2 in abs. alcohol). It is easily soluble in alcohol, ether and hot water, sparingly soluble in cold water and dilute mineral acids and almost insoluble in light petroleum. It possesses one molecule of water of crystallization after drying at room temperatures over CaCl₂. (Found: C, 54.6; H, 8.35; H₂O, 5.3%; equiv. by titration, 350.4. C₁₆H₂₈O₇, H₂O requires C, 54.8; H, 8.6; H₂O, 5.1%; equiv. 350.2.)

On keeping the filtrate after separating *d*-menthylglucuronide as above for several days, a second crop of crystals was obtained. These consisted of minute needles and had $[\alpha]_D - 44^\circ$ in water (m.p. 107–109°). They contained glucuronic acid and on hydrolysis gave a terpene alcohol with a menthol-like odour. They have not been identified so far.

Ammonium-d-menthylglucuronate. A pure specimen of this salt was prepared by dissolving the pure *d*-acid in water and neutralizing with a few drops of ammonia (sp. gr. 0.880). On keeping the solution the salt separated as needles, melting with decomposition at 200–202° and soluble in warm alcohol ($[\alpha]_D^{20} + 8.1^\circ$, *c* = 1 in water). (Found: N, 3.8; H₂O, 5.2%. C₁₆H₂₇O₇NH₄, H₂O requires N, 3.8; H₂O, 4.9%.)

Preparation of d-menthol. Acid hydrolysis of the *d*-salt obtained directly from the resolutions does not give an optically pure menthol owing to the presence of an unknown terpene alcohol already referred to above. A specimen obtained in this manner had m.p. 34–36° and $[\alpha]_D^{15} + 46.4^\circ$ in alcohol. An optically pure *d*-menthol was obtained on hydrolysis of pure *d*-menthylglucuronide. The glucuronide (2 g.) was hydrolysed by steam distillation with 15 ml. 2*N* H₂SO₄. The menthol in the distillate was extracted with light petroleum, the solution filtered and evaporated. The *d*-menthol formed long needles (yield 0.85 g., almost quantitative) and had a fainter odour than its *l*-enantiomorph as has been observed by previous investigators. It had m.p. 41° (corr.) and

¹ The figures quoted in this table were obtained by Dr M. Stacey during the preparation of glucurone from menthylglucuronide prepared by the author.

$[\alpha]_D^{20} + 49.4^\circ$ ($c = 1.66$ in abs. alcohol; mean of three determinations). Read & Grubb [1934] give for the *d*- and *l*-menthols, M.P. $42-43^\circ$ and $[\alpha]_D \pm 49.6^\circ$ in alcohol.

Two new derivatives of *d*-menthol were prepared for comparison with the known *l*-derivatives. *d*-Menthylphenylurethane (*d*-menthyl carbanilate) was prepared by heating *d*-menthol with phenylisocyanate. It forms long silky needles from light petroleum, M.P. $112-113^\circ$ (corr.) and $[\alpha]_D^{20} + 75.7^\circ$ ($c = 1$, in CHCl_3). (Found: N, 5.3 %. $\text{C}_{17}\text{H}_{25}\text{O}_2\text{N}$ requires N, 5.1 %.) Kenyon & Pickard [1915] give $[\alpha]_D - 76.9^\circ$ (in CHCl_3) for the *l*-form. *d*-Menthyl-3:5-dinitrobenzoate was prepared from *d*-menthol and 3:5-dinitrobenzoyl chloride. It forms long fluffy needles from alcohol and has M.P. $153-154^\circ$ (corr.) and $[\alpha]_D^{20} + 77.1^\circ$ ($c = 0.8$, in CHCl_3). (Found: N, 7.7 %. $\text{C}_{17}\text{H}_{22}\text{O}_6\text{N}_2$ requires N, 8.0 %.) Read *et al.* [1933] give M.P. 153° and $[\alpha]_D - 79.4^\circ$ (in CHCl_3) for the *l*-derivative.

SUMMARY

The optical resolution of *dl*-menthol has been effected through its conjugation with glucuronic acid in the rabbit. Two methods were used:

(a) The ammonium salts of the *d*- and *l*-menthylglucuronides, excreted in the urine following the oral administration of *dl*-menthol, were fractionally crystallized.

(b) *l*-Menthol was eliminated by taking advantage of its greater ease of oxidation in the rabbit than *d*-menthol. By re-feeding menthol recovered from a previous administration of *dl*-menthol, nearly 90 % of the *l*-menthol was oxidized biologically and the excreted glucuronide was mainly *d*-menthylglucuronide which was easily purified.

d-Menthol, from hydrolysis of *d*-menthylglucuronide, was obtained in about 10 % yield, calculated on the *dl*-menthol fed. *l*-Menthol was not readily obtainable in optically pure form by these methods.

The excretion of conjugated menthol, following a dose of 3 g. per rabbit, is complete in about 30 hr., the material excreted throughout this period having a constant ratio of *d*- to *l*-menthol.

The following derivatives of *d*-menthol are described for the first time: *d*-menthylglucuronide and its ammonium salt, *d*-menthylphenylurethane and *d*-menthyl 3:5-dinitrobenzoate.

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