84. STUDIES IN DETOXICATION

7. THE BIOLOGICAL REDUCTION OF *l*-MENTHONE TO *d*-*NEO*MENTHOL AND OF *d*-*ISO*MENTHONE TO *d*-*ISO*MENTHOL IN THE RABBIT. THE CONJUGATION OF *d*-*NEO*MENTHOL WITH GLUCURONIC ACID

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A survey of the literature has shown that foreign ketones, when considered in relation to their fate in the mammalian organism, may be divided into two main groups.

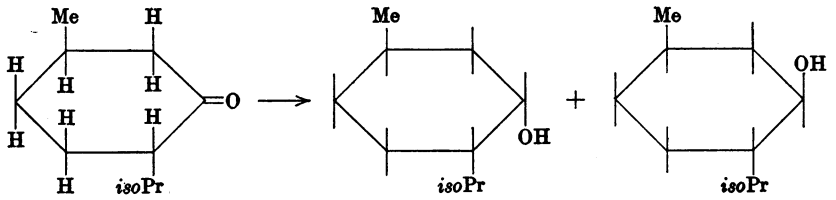
(1) *Aliphatic and aromatic ketones.* These undergo both oxidation and reduction in the body. As a result of biological reduction of the carbonyl group, secondary alcohols are produced which are usually excreted in conjugation with glucuronic acid. It should be noted that in these ketones the carbonyl group is the main point at which the organism attacks the molecule. Several aromatic ketones have been studied [cf. Thierfelder & Daiber, 1923] and acetophenone may be taken as a typical example. Besides being oxidized to benzoic acid [Nencki, 1878; Quick, 1928] and other products, a considerable proportion (36%) of it is reduced to methylphenylcarbinol [Thierfelder & Daiber, 1923]. Very few aliphatic ketones have been studied [cf. Neubauer, 1901], but Saneyoshi [1911] has shown that methylethylketone is reduced in rabbits, the reduction product, 2-butanol, being isolated from the urine as the glucuronide. *cyclo*-Hexanone is oxidized to adipic acid [Filippi, 1914; Bernhard, 1937].

(2) *Terpene ketones.* The ketones of this group that have been studied are all derivatives of bicyclic terpenes; very little work has been done on the monocyclic ketones. Unlike the ketones of the aliphatic and aromatic type, the carbonyl group of the bicyclic terpene ketones or camphors is very resistant to oxidation or reduction in the mammalian organism and usually survives unchanged, oxidation taking place elsewhere in the molecule with the production of a keto-alcohol which is usually excreted in conjugation with glucuronic acid. The following bicyclic ketones have been shown to behave in this manner, thujone [Hämäläinen, 1912; Hildebrandt, 1901], carone [Rimini, 1901], *d*-camphor [Asahina & Ishidate, 1933; 1934; 1935], *l*-camphor [Magnus-Levy, 1906], *r*-camphor [Mayer, 1908], *epi*camphor [Reinartz *et al.* 1934, 1], 5-oxocamphor [Reinartz *et al.* 1934, 2], dimethylcamphor [Reinartz & Meessen, 1939], *d*-fenchone [Reinartz & Zanke, 1936] and α-santenone (*π-nor*camphor) [Hämäläinen, 1912]. The only exception found so far is the camphane derivative, camphenilone, which was shown by Hämäläinen [1912] to undergo, in the rabbit, reduction of the keto group with the formation of camphenilol.

The fate of l-*menthone*

Three monocyclic terpene ketones have been studied without, however, any clear results. Hildebrandt [1902] showed that pulegone and carvone produced *in vivo* conjugated glucuronides of undetermined nature and Teppati [1937] claimed that pulegone was reduced to *d*-menthol and *l*-pulegol, although his evidence was unconvincing. Bonnani [1902] and Neubauer [1901] both demonstrated qualitatively that *l*-menthone gave rise, in rabbits, to a conjugated glucuronic acid. Later, Hämäläinen [1912] stated that *l*-menthone was oxidized in the rabbit to a tertiary keto-alcohol, probably 4-hydroxymenthone, but the evidence offered was inconclusive.

In the present paper definite evidence is presented to show that at least part of the *l*-menthone molecule undergoes reduction at the carbonyl group with the production of *d-neo*menthol which is excreted as a glucuronide. This glucuronide was isolated as the ammonium salt and, judging from the yield of this salt, at least 10–15 % of the *l*-menthone fed was reduced to *d-neo*menthol. Quantitative measurements of the glucuronic acid present in rabbit urine after *l*-menthone (see Table 1) showed that about 30–40 % of the menthone fed was converted into hydroxy derivatives which were excreted in conjugation with glucuronic acid. It has been shown by Read & Grubb [1934, 1] that reduction of *l*-menthone (I) with Ponndorf's reagent produced *d-neo*menthol (II) and *l*-menthol (III) in amounts of 70 and 30 % respectively. It is therefore possible that the reduction of



In the above formulae, the configurations used are those put forward by Read & Grubb [1934, 2].

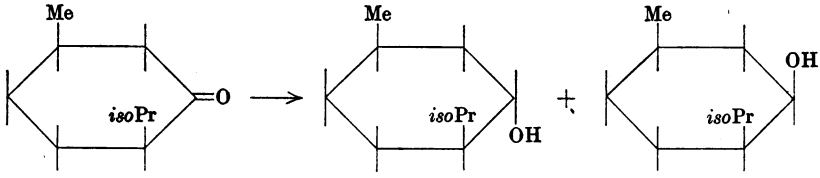
l-menthone *in vivo* would produce *l*-menthol in addition to *d-neo*menthol. *l*-Menthol, however, was not found, although its detection would have been a simple matter since its glucuronide has a high negative rotation ($[\alpha]_D - 110°$) compared with the other menthylglucuronides (see Williams [1939] for table of rotations). The possible explanations for the absence of *l*-menthylglucuronide other than experimental error, are (1) during the biological reduction of *l*-menthone, the production of the new optically active carbon atom is a completely asymmetric process, *d-neo*menthol only being formed, and (2) the biological reduction produces both menthanols (and here the process may be partially asymmetric or completely symmetric) but the rate of oxidation of *l*-menthol or its glucuronide is greater than that of *d-neo*menthol or its glucuronide and is such that no *l*-menthol survives at all. Some support is to be found for the second of these explanations, inasmuch as 67–68 % of *d-neo*menthol, when fed to a rabbit, is excreted in conjugation with glucuronic acid (see Table 3), whilst only 48 % of *l*-menthol is so conjugated [Williams, 1938]. It is not yet clear whether the oxidation and conjugation of menthols in the body are simultaneous or consecutive processes. Conjugation with glucuronic acid might well be an

intermediate stage in the biological oxidation of menthols. This point, however, is under investigation.

It appears, therefore, that *l*-menthone in its behaviour *in vivo* resembles the "aliphatic-aromatic" ketones since it undergoes reduction of its carbonyl group producing the secondary alcohol *d-neo*menthol. It may also resemble the camphors if Hämäläinen's [1912] claim, that it forms a keto-alcohol in which the original keto group still survives, can be substantiated.

The fate of d-iso*menthone*

Reduction of *d-iso*menthone (IV) should give *d-iso*menthol (V) and *d-neoiso*menthol (VI) [Read & Grubb, 1934, 3]. Hückel & Niggemeyer [1939] have shown that hydrogenation of *d-iso*menthone in acid solution in the presence of a Pt catalyst gave *d-neoiso*menthol, no *d-iso*menthol being formed. It would



IV V VI

be interesting to see what would happen to this ketone during biological reduction. A limited amount of *d-iso*menthone became available through the courtesy of Messrs Howard, Ltd., Ilford. Read & Grubb [1934, 3] state that *d-iso*menthone undergoes inversion to some extent into *l*-menthone; the material used in these experiments showed $[\alpha]_D + 78°$ compared with $+92°$ for the pure material, so that the ketone fed contained about 75% *d-iso*menthone, the rest being, presumably, *l*-menthone. On feeding 24 g. (dose 3 g./rabbit) of this ketone, 2·8 g. of crude ammonium menthanolglucuronate were isolated from the urine. The only substance definitely identified in this salt was *d-iso*menthylglucuronide which seemed to be the major if not the only substance present (this glucuronide has been previously described in Parts 2 and 4 of this series [Williams, 1938; 1940, 1]). It was hoped that the unknown *d-neoiso*menthylglucuronide would be isolated, but a thorough search for this compound could not be carried out owing to scarcity of *iso*menthone. It is hoped that the fate of this ketone can be reinvestigated at a later date when more material becomes available. However, on the basis of this single experiment it can be definitely stated that, like *l*-menthone, at least part of the *d-iso*menthone molecule is reduced in the rabbit to menthanols one of which is *d-iso*menthol.

The fate of d-neo*menthol*

It is shown in the present work that as far as that portion of *d-neo*menthol which undergoes conjugation with glucuronic acid is concerned, 67–68% of the material fed is conjugated. It has been shown in an earlier paper [Williams, 1938] that the *d*-menthanols, *d*-menthol and *d-iso*menthol, conjugate with glucuronic acid in the rabbit to the extent of 70 and 65% respectively, whereas only 48 and 45% respectively of the corresponding *l*-derivatives are so conjugated. The extent of conjugation of *d-neo*menthol is therefore of the same order as that of *d*-menthol and *d-iso*menthol. These three *d*-menthanols are structural isomerides (II, III, V) and it appears that this structural variation has no influence on their

conjugation with glucuronic acid in the body, although in purely chemical reactions such as the rate of formation of *p*-nitrobenzoates they show considerable differences [see Read & Grubb, 1934, 2]. Their *l*-antipodes conjugate to a much smaller extent with glucuronic acid [Williams, 1938].

EXPERIMENTAL

Experiments with l-*menthone*

Isolation of the conjugated glucuronide. Rabbits (2–3 kg.) on a diet of 100 g. cabbage and 50 g. bran a day were given 3 g. (3·3 ml.) of *l*-menthone[1] by stomach tube with water. The urine was collected during 2 days and precipitated with $(NH_4)_2SO_4$ (50 g./100 ml. urine) as described in earlier papers [e.g. Williams, 1940, 2]. The ammonium sulphate-treated urine was kept at 0° for 2 days and, during that time, a crystalline precipitate gradually settled, which proved to be crude *ammonium* d-neo*menthylglucuronate*. Various specimens of the crude dry salt showed $[\alpha]_D - 8$ to $-10°$ in water and the yield was 0·28–0·3 g./g. of *l*-menthone fed; a total of 14·4 g. of the salt was isolated after feeding 57 g. of the ketone. The urine after separation of the salt was made strongly acid with 20 % H_2SO_4 and thoroughly extracted with ether. The ethereal extract on evaporation left a clear brown syrup from which no crystalline material could be isolated. This syrup was mainly composed of glucuronides since it gave a very intense naphthoresorcinol reaction and smelt strongly of terpenes. Treatment of a portion of the syrup dissolved in alcohol with 2:4-dinitrophenylhydrazine gave a small amount of an unidentified hydrazone, but no *l*-menthone 2:4-dinitrophenylhydrazone was isolated. The syrup decolorized permanganate, but no definite evidence as to its composition has yet been obtained.

Isolation of d-neo*menthylglucuronide.* The above ammonium salt (4·5 g.) was dissolved in 200 ml. of water and the solution warmed and filtered. To the filtrate 15 ml. of $2N$ H_2SO_4 were added and a crystalline precipitate (3 g.) was immediately formed. A further quantity was obtained from the mother liquor by ether extraction. No *l*-menthylglucuronide was detected in the mother liquor. The above crystalline material after several recrystallizations from hot water was obtained pure and had M.P. 145° unchanged in mixture with authentic d-neo*menthylglucuronide* (see below). It formed long slender white needles, sparingly soluble in cold water but easily soluble in hot water, alcohol and ether; it showed $[\alpha]_D^{23°} - 15°$ ($c = 1·4$ in alcohol) and $[\alpha]_D - 10°$ ($c = 1$ in $0·8N$ NaOH). (Found: C, 54·9; H, 8·7; H_2O, 5·1 %; equiv. by titration, 349. $C_{16}H_{28}O_7$, H_2O requires C, 54·8; H, 8·6; H_2O, 5·1 %; equiv. 350.)

Hydrolysis of the glucuronide: isolation of d-neo*menthol.* The foregoing glucuronide (2 g.) was hydrolysed by steam-distilling with 20 ml. of $0·5N$ H_2SO_4. The *neo*menthol in the distillate was extracted with light petroleum and the extract dried with anhydrous Na_2SO_4. Evaporation of the petroleum left *d-neo*menthol as a colourless thick liquid (0·93 g.) with $n_D^{14°} = 1·4617$ and $[\alpha]_D^{22°} + 19·8°$ ($c = 2·3$ in alcohol); Read & Grubb [1934, 1] give $n_D^{17°} = 1·4617$ and $[\alpha]_D + 20·7°$ (in alcohol). It was further identified as the crystalline *p*-nitrobenzoate and 3:5-*dinitrobenzoate*. *d-neo*Menthyl *p*-nitrobenzoate from the above biosynthetic *d-neo*menthol showed $[\alpha]_D^{22°} + 18·96°$ ($c = 1$ in $CHCl_3$) and had M.P. 94° unchanged in mixture with an authentic specimen; Read & Grubb [1934, 1] give M.P. 94·5–95° and $[\alpha]_D + 17·8°$ (in $CHCl_3$). d-neo*Menthyl* 3:5-*dinitrobenzoate* prepared

[1] Howard, Ltd., Ilford. The menthone showed $[\alpha]_D^{14°} - 25·3°$ ($c = 2$ in alcohol) and was free from menthols, since no menthyl *p*-nitrobenzoates could be isolated from it on heating with *p*-nitrobenzoyl chloride in the presence of pyridine in the usual manner.

from the biosynthetic *neo*menthol formed long needles and after three recrystallizations from alcohol had M.P. 155° and showed $[\alpha]_D^{22°}+23{\cdot}6°$ ($c=0{\cdot}9$ in $CHCl_3$). (Found: C, 58·1; H, 6·1; N, 8·15%. $C_{17}H_{22}N_2O$ requires C, 58·25; H, 6·3; N, 8·0%.) Since this dinitrobenzoate has not been previously described an authentic specimen was prepared by condensing *d-neo*menthol (Eastman Kodak, Ltd.) with 3:5-dinitrobenzoyl chloride in the presence of pyridine in the usual manner. The authentic material formed long needles from alcohol, M.P. 155° (not depressed by admixture with the dinitrobenzoate from biosynthetic *d-neo*menthol) and $[\alpha]_D^{22°}+24{\cdot}8°$ ($c=0{\cdot}95$ in $CHCl_3$). Read & Grubb [1933] give M.P. 153° and $[\alpha]_D-23{\cdot}9°$ (in $CHCl_3$) for *l-neo*menthyl 3:5-dinitrobenzoate.

Quantitative determination of glucuronic acid in urine after feeding menthone. Rabbits on a standard diet were given *l*-menthone (dose 1 g./kg.) with water by stomach tube. The urine was collected for about 40 hr. and analysed for glucuronic acid by an adaptation of the Shaffer-Hartmann blood sugar method. For this purpose 1 ml. of the filtered urine was mixed with 1 ml. of 2% H_2SO_4 and extracted for 1 hr. with ether in a small (2 ml.) urine extractor. The ethereal extract was evaporated and the residue hydrolysed by boiling for 10 min. on a hot-plate with 5 ml. *N* HCl. The cooled solution was then exactly neutralized with *N* NaOH using a trace of methyl red as indicator (a drop from a capillary tube). The evaporation, hydrolysis and neutralization can be carried out in the boiling flask of the micro-extractor. The solution was now transferred quantitatively to a 25 ml.[1] measuring flask and made up to the mark. The glucuronic acid in this solution was determined on 5 ml. portions using the Shaffer-Hartmann reagent 50 of Shaffer & Somogyi [1933] (containing 150 ml. of KIO_3 solution equivalent to 0·1 *N* I_2). The glucurone content of the solution was then read off from a graph previously constructed from titrations of standard solutions of pure glucurone and sodium glucuronate. It was found that solutions of glucurone exactly neutralized in the cold with NaOH (i.e. sodium glucuronate) had the same reducing power as the equivalent of glucurone. The results for *l*-menthone are given in Table 1.

Table 1. *Glucuronic acid conjugation in the rabbit after* l-*menthone*

Rabbit no.	Wt. of rabbit, kg. and dose g.	Time of urine collection hr.	Glucurone found g.	Menthone conjugated g.	Menthone converted into hydroxy compounds %
61	2·0	43	0·9559	0·8254	41·3
62	2·4	43	0·8976	0·7752	32·3
63	2·6	43	0·8550	0·7384	28·4
61	2·0	48	0·9048	0·7918	39·6

Experiments with d-iso*menthone*

Isolation of an ammonium menthylglucuronate. Eight rabbits were each given 3 g. of *d-iso*menthone ($[\alpha]_D+78°$; $c=2$ in alcohol) with water by stomach tube. The urine was collected during 2 days and treated with $(NH_4)_2SO_4$ as previously described. After 2 days at 0° the urine contained a small amount of crystalline precipitate which was collected and dried (yield, 2·8 g.). The crude salt gave an intense naphthoresorcinol reaction and showed $[\alpha]_D-16{\cdot}3°$ ($c=1$ in water).

Isolation and identification of d-iso*menthylglucuronide.* The whole of the above salt was dissolved in 50 ml. water and the solution filtered and acidified with dilute H_2SO_4. On standing the solution deposited a nodular solid which was

[1] The dilution is so arranged that 5 ml. of the solution contain 0·5–1·5 mg. of glucurone.

coloured owing to adsorption of urinary pigments. The solid was recrystallized 4 times from hot water and eventually typical long white needles of *d-iso*menthylglucuronide were obtained, M.P. 124–125° and mixed M.P. 125–126° and showing $[\alpha]_D^{22°}$ −42·9° (c=1 in alcohol) (Williams [1938] gives M.P. 126° and $[\alpha]_D$ −43·2° in alcohol). It was further identified by isolation of *d-iso*menthol. A small amount of the glucuronide was hydrolysed by dilute acid and the *iso*menthol formed recovered by a simultaneous steam-distillation. The recovered *d-iso*menthol had M.P. 80° and mixed M.P. 80–81° (authentic *d-iso*menthol has M.P. 82–83°).

Experiments with d-neo*menthol*

Ammonium d-neo*menthylglucuronate.* Two rabbits were each given 3 g. (3·3 ml.) of *d-neo*menthol ($[\alpha]_D^{22°}$ +22°, c=2 in alcohol) (Eastman Kodak, Ltd.) with water by stomach tube. The urine was collected during 2 days and crude ammonium *d-neo*menthylglucuronate (5·2 g.) isolated from it by the ammonium sulphate method [cf. Williams, 1940, 2]. The crude salt showed $[\alpha]_D^{20°}$ −5·2° (c=2 in water). It was dissolved in the minimum of water with warming and the solution was filtered. Acetone (2–3 vol.) was then added whereby a small precipitate was formed. This was discarded since it was mainly $(NH_4)_2SO_4$ and acetone was now added to the solution in excess thereby precipitating *ammonium* d-neo*menthylglucuronate* in an apparently amorphous state. On standing, the salt crystallized and was then recrystallized from aqueous acetone. The salt formed long matted needles and after drying *in vacuo* over $CaCl_2$ contained 1 mol. H_2O. The monohydrate showed $[\alpha]_D$ −5·9° (c=2 in water) and the anhydrous salt (dried at 110°) showed $[\alpha]_D^{20°}$ −6·9° (c=2 in water). (Found: glucurone by Shaffer-Hartman, 47·7; H_2O, 4·6%. $C_{16}H_{27}O_7NH_4, H_2O$ requires glucurone, 47·9; H_2O, 4·9%.)

Preparation of d-neo*menthylglucuronide.* (*a*) Rabbits were given *d-neo*menthol as described above. After 2 days the urine was collected, filtered through muslin and treated with 1/20 vol. of 20% H_2SO_4. It was kept at 0° for about an hour and during that time *d-neo*menthylglucuronide separated as long needles. The crystals were filtered off under suction and dried in a desiccator. The yields of the crude acid are given in Table 2; only about 50% of the acid in the urine could be recovered in this manner and the greater the volume of fluid the less was the yield.

Table 2. *Preparation of* d-neo*menthylglucuronide*

Rabbit no.	Dose g.	Time of urine collection hr.	Vol. of urine + washings ml.	Yield of crude acid g.
59, 60	3	48	600	3·2
67, 70	3	48	510	3·8
68, 69	3	48	480	4·1
56	2·4	24	164	1·5

(*b*) An aqueous solution of the ammonium salt was acidified with dilute H_2SO_4 and *d-neo*menthylglucuronide immediately separated as fine felted needles. After two recrystallizations from water, the acid had M.P. 146° and showed $[\alpha]_D^{22°}$ −14·6° (c=2 in alcohol).

Quantitative determination of glucuronic acid in urine after d-neo*menthol.* Rabbits were given *d-neo*menthol (dose 1 g./kg.) with water by stomach tube.

The urine collected was analysed for glucuronic acid by the Shaffer-Hartmann method as described above in the section on *l*-menthone. The results are given in Table 3.

Table 3. *The conjugation of* d-neo*menthol with glucuronic acid in the rabbit*

Rabbit no.	Wt. of rabbit, kg. and dose g.	Time of urine collection hr.	Glucurone found g.	*neo*Menthol conjugated g.	% *neo*Menthol conjugated
56	2·38	24	1·427	1·248	52·4
61	2·02	43	1·582	1·384	68·5
64	2·23	43	1·714	1·499	67·2
66	2·50	43	1·915	1·675	67·0

Summary

The fate of *l*-menthone, *d-iso*menthone and *d-neo*menthol in the rabbit has been studied.

It has been found that on administration of *l*-menthone to rabbits, about 30–40 % of it is excreted as hydroxy derivatives conjugated with glucuronic acid. Isolation of *d-neo*menthylglucuronide from the urine indicates that at least part of the menthone molecule is reduced at the carbonyl group. About 10–15 % of the *l*-menthone fed was actually isolated as *d-neo*menthylglucuronide.

*d-iso*Menthone is also reduced in the rabbit, *d-iso*menthol (isolated as the glucuronide) being identified as a reduction product.

On feeding *d-neo*menthol to rabbits, 67–68 % was excreted in the urine combined with glucuronic acid; this figure is of the same order as those previously found for *d*-menthol (70 %) and *d-iso*menthol (65 %).

A method is described, using a Shaffer-Hartmann reagent, for the quantitative estimation of conjugated glucuronic acid in 1 ml. urine after feeding menthol derivatives.

The following compounds are described for the first time: *d-neo*menthylglucuronide and its ammonium salt and *d-neo*menthyl 3:5-dinitrobenzoate.

REFERENCES

Asahina & Ishidate (1933). *Ber. dtsch. chem. Ges.* **66**, 1673.
—— —— (1934). *Ber. dtsch. chem. Ges.* **67**, 71.
—— —— (1935). *Ber. dtsch. chem. Ges.* **68**, 947.
Bernhard (1937). *Hoppe-Seyl. Z.* **248**, 256.
Bonnani (1902). *Arch. Farmacol. sper.* **1**, 469, 498.
Filippi (1914). *Arch. Farmacol. sper.* **18**, 178 (cited by Bernhard, 1937).
Hämäläinen (1912). *Skand. Arch. Physiol.* **27**, 141.
Hildebrandt (1901). *Arch. exp. Path. Pharmak.* **45**, 110.
—— (1902). *Hoppe-Seyl. Z.* **34**, 456.
Hückel & Niggemeyer (1939). *Ber. dtsch. chem. Ges.* **72**, 1354.
Magnus-Levy (1906). *Biochem. Z.* **2**, 319.
Mayer (1908). *Biochem. Z.* **9**, 439.
Nencki (1878). *J. prakt. Chem.* **18**, 288.
Neubauer (1901). *Arch. exp. Path. Pharmak.* **46**, 152.
Quick (1928). *J. biol. Chem.* **80**, 515.

Read & Grubb (1933). *J. chem. Soc.* p. 167.
—— —— (1934, 1). *J. Soc. chem. Ind., Lond.*, **53**, 52 T.
—— —— (1934, 2). *J. chem. Soc.* p. 1779.
—— —— (1934, 3). *J. chem. Soc.* p. 316.
Reinartz & Meessen (1939). *Ber. dtsch. chem. Ges.* **72**, 1.
—— & Zanke (1936). *Ber. dtsch. chem. Ges.* **69**, 2259.
—— —— & Faust (1934, 1). *Ber. dtsch. chem. Ges.* **67**, 589.
—— —— —— & Schaefers (1934, 2). *Ber. dtsch. chem. Ges.* **67**, 1536.
Rimini (1901). *Chem. Z.* **1**, 1227 (cited by Hämäläinen, 1912).
Saneyoshi (1911). *Biochem. Z.* **36**, 22.
Shaffer & Somogyi (1933). *J. biol. Chem.* **100**, 695.
Teppati (1937). *Arch. int. Pharmacodyn.* **57**, 440.
Thierfelder & Daiber (1923). *Hoppe-Seyl. Z.* **130**, 380.
Williams (1938). *Biochem. J.* **32**, 1849.
—— (1939). *Biochem. J.* **33**, 1519.
—— (1940, 1). *Biochem. J.* **34**, 48.
—— (1940, 2). *Biochem. J.* **34**, 272.