Studies in Detoxication

47. THE FORMATION OF ESTER GLUCURONIDES OF ALIPHATIC ACIDS DURING THE METABOLISM OF 2-ETHYLBUTANOL AND 2-ETHYLHEXANOL

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The octyl alcohol, DL-2-ethyl-*n*-hexanol, is used industrially as a solvent and, in the form of the ester, di-2-ethylhexyl phthalate, as a plasticizer. According to Shaffer, Carpenter & Smyth (1945) this ester is saponified in rats and rabbits, phthalic acid being excreted and there being also an increase in the short-chain fatty acids in the urine. After feeding the DL-alcohol to rabbits, Akiya & Yamakawa (1950) isolated α -ethylhexanoic acid (α -ethylcaproic) from the urine, showing a slight positive rotation. They also found a dibasic acid with a slight positive rotation. No reports of the formation of glucuronide from the alcohol have been made. The toxicity of 2-ethylhexanol is comparable with that of *iso*octyl alcohol, the anaesthetic dose of both alcohols being near the lethal dose in rats (Nelson, 1951).

It has thus been proved that 2-ethylhexanol is oxidized *in vivo* to the corresponding fatty acid and it would be expected that 2-ethylbutanol would be similarly oxidized to diethylacetic acid (α -ethylbutyric acid). Blum & Koppel (1911) injected diethylacetic acid into a dog and reported methyl *n*-propyl ketone in the urine. Dziewiatkowski, Venkataraman & Lewis (1949) also fed and injected this acid into rats and rabbits, but were unable to detect any ketone in the urine. They did, however, observe that both diethylacetic and 2-ethylhexanoic acids gave rise to copper-reducing urines and extra glucuronic acid. This suggested that these acids were being excreted partly as ester glucuronides. In the rabbit extra glucuronic acid corresponding to 25–52% of the dose of diethylacetic acid and to 83–87% of the α -ethylhexanoic acid was excreted. The ester glucuronides, however, were not isolated.

In the present work it has been found that 2-ethylbutanol and 2-ethylhexanol give rise in the rabbit to the excretion of the ester glucuronides of diethylacetic and α -ethylhexanoic acids respectively. These glucuronides have been isolated and appear to be the first ester glucuronides of fatty acids to be described.

EXPERIMENTAL

In the preceding paper (Kamil, Smith & Williams, 1953) it was shown that extra glucuronic acid was excreted by rabbits after dosing with 2-ethylbutanol

and 2-ethylhexanol. The glucuronic acid excretion corresponded to 40% of the dose of 2-ethylbutanol and 87% of 2-ethylhexanol (dose 25 m-moles/3 kg. rabbit).

Metabolism of 2-ethylbutan-1-ol

Isolation of diethylacetic acid and methyl propyl ketone. A rabbit was given 2.55 g. (3.1 ml.) of the alcohol and a 24 hr. urine was collected. This urine was strongly reducing and gave a strong positive naphthoresorcinol test which indicated the presence of a reducing ester glucuronide. The urine was acidified, treated with 100 ml. of Brady's reagent and kept for 24 hr. The precipitate which had formed was filtered and recrystallized from ethyl acetate. It proved to be methyl *n*-propyl ketone 2:4-dinitrophenylhydrazone (yield, 7 mg.), m.p. and mixed m.p. 140°. (Found: N, 20.6. Calc. for $C_{11}H_{14}N_4O_4$: N, 21.0%.) The filtrate from the hydrazone was made 2N with respect to Na_2CO_3 by adding solid Na_2CO_3 , and then boiled for 0.5 hr. After cooling, the solution was acidified with H_2SO_4 and extracted in a funnel with 2 x 200 ml. of ether. The extract was dried over $CaCl_2$, evaporated and the oily residue distilled. The fraction boiling at 140–200° was collected and heated with an equal weight of thionyl chloride till the reaction ceased. The product was then treated with an equal weight of *p*-toluidine, warmed and the product recrystallized from a large volume of hot water. In this way, 0.5 g. of the *p*-toluidide of diethylacetic acid, m.p. and mixed m.p. 117° (m.p. 116.2° is quoted by Huntress & Mulliken, 1946), was isolated. (Found: C, 75.6; H, 9.4; N, 7.0. Calc. for $C_{13}H_{16}NO$: C, 76.0; H, 9.3; N, 6.8%.)

Isolation of diethylacetylglucuronide. From the 24 hr. urine of four rabbits which had collectively received 14 ml. of diethylethanol, there were obtained through the basic lead salt 13 g. of glucuronide gum which could not be crystallized. The potassium salt of β -diethylacetylglucuronide (0.25 g.) was obtained in poor yield by mixing 1.2 g. of the gum, 70 mg. K_2CO_3 and a few drops of water, then diluting with a few ml. of ethanol and filtering. The filtrate was diluted to 50 ml. with 98% ethanol and on keeping at 0°, the potassium salt separated as

rectangular plates which reduced Fehling's solution on boiling. These decomposed at 168–170° and showed $[\alpha]_D^{20} - 27.1^\circ$ in water (c, 0.8). (Found: C, 41.5; H, 6.2; K, 12.0; glucuronic acid, $C_6H_{10}O_7$, 52.3. $C_{12}H_{18}O_8 \cdot K \cdot H_2O$ requires C, 41.4; H, 6.0; K, 11.2; $C_6H_{10}O_7$, 55.7%.)

The gum also yielded on methylation and acetylation *triacetyl β-diethylacetyl-D-glucuronide methyl ester* in poor yield. This formed long fine needles from *n*-hexane; it was optically inactive in $CHCl_3$ and had m.p. 72°. (Found: C, 52.9; H, 6.5. $C_{19}H_{28}O_{11}$ requires C, 52.7; H, 6.5%.) The compound was insoluble in water and reduced Benedict's reagent on heating when acetone was added to assist its solution.

The metabolism of DL-2-ethylhexan-1-ol

The urine of rabbits receiving 2-ethylhexanol strongly reduced copper reagents and this, together with an intense naphthoresorcinol reaction, suggested the formation of an ester glucuronide which was isolated.

Isolation of α-ethylhexanoylglucuronide. Three rabbits were each given 3 ml. of 2-ethylhexanol by stomach tube. The 24 hr. urine was acidified to pH 4 with HCl and continuously extracted with ether for 8 hr., when the extraction was complete. Evaporation of the ether gave the glucuronide gum (5 g.). This was treated with 1 g. of K_2CO_3 and when effervescence had ceased the residue was dissolved in warm ethanol (50 ml.). On standing, *potassium β-2-ethylhexanoyl-D-glucuronide monohydrate* separated (2 g.). It formed white needles from ethanol, m.p. 170° decomp. and $[\alpha]_D - 27.5^\circ$ in water (c, 0.6). (Found: C, 44.2; H, 6.5; K, 10.6. $C_{14}H_{23}O_8 \cdot K \cdot H_2O$ requires C, 44.7; H, 6.7; K, 10.4%.) It reduced Benedict's and Fehling's solution on heating. *Triacetyl β-2-ethylhexanoyl-D-glucuronide methyl ester* formed colourless needles from hexane, m.p. 82°. (Found: C, 55.2; H, 7.0. $C_{21}H_{32}O_{11}$ requires C, 54.8; H, 7.0%.) This ester was optically inactive in chloroform and reduced Benedict's solution on heating with a little acetone to assist solution.

Isolation of α-ethylhexanoic acid. Three rabbits were given 3 ml. each of 2-ethylhexanol and the 24 hr. urine (260 ml.) treated with 8 ml. conc. HCl. The solution was heated to boiling for 15 min., cooled and extracted with ether (250 ml.). The extract, which reduced Benedict's reagent, was evaporated and the residue dissolved in water. α-Ethylhexanoic acid was extracted with light petroleum (b.p. 40–60°) in which the glucuronide is insoluble. The light petroleum was evaporated and the residual oil distilled. The main fraction (1 ml.) distilled at 225° and the undiluted oil showed $[\alpha]_D^{20} - 1.2^\circ \pm 0.05$. The α-ethylhexanoic acid was thus optically active. The optical isomers of α-ethylhexanoic acid have been prepared by Kenyon

& Platt (1939) who record $[\alpha]_D^{20} + 8.95^\circ$ and -8.75° . It would thus appear that our material contains roughly 56% (–) isomer and 44% (+) isomer. On treatment with thionyl chloride followed by ammonium hydroxide the acid yielded crystalline α-ethyl-*n*-hexanoamide, m.p. 103–104° (from ligroin), mixed m.p. 104°, with authentic α-ethyl-*n*-hexanoamide, m.p. 104–105° (Magnani & McElvain, 1938).

2-Ethylhexanol urine also contained traces of a ketone, which could be di-*n*-propyl ketone or *n*-amyl methyl ketone, but the amounts present were too small for satisfactory identification. An attempt was made to detect this ketone in the breath of rabbits. A rabbit was given 6 ml. of 2-ethylhexanol and placed in the Perspex tank mentioned earlier. The expired air was drawn through Brady's reagent for 24 hr. but no ketone was detected.

DISCUSSION

In the preceding paper (Kamil *et al.* 1953) it was suggested that the glucuronic acid conjugation of primary aliphatic alcohols was low, probably because of their rapid oxidation. The glucuronic acid excretion after feeding 2-ethylbutanol and 2-ethylhexanol is, however, very high and it has been shown that this is due, not to the conjugation of the alcohols themselves with glucuronic acid, but to the conjugation of their oxidation products. The formation and isolation of the ester glucuronides of diethylacetic and α-ethylhexanoic acids after feeding the corresponding alcohols thus shows that the primary aliphatic alcohols are rapidly oxidized *in vivo* and that the low percentage of ether glucuronide formation is due to the rapidity of this oxidation.

In all we have studied four 2-substituted primary alcohols; two of them, isobutanol and 2-methylbutanol, carry 2-methyl groups and are of the general type $RCH(CH_3)CH_2OH$, whereas the other two, 2-ethylbutanol and 2-ethylhexanol, carry 2-ethyl groups and are of the general type

The 2-methyl-substituted alcohols should give rise to α-methyl fatty acids, $RCH(CH_3)COOH$, which are readily oxidized *in vivo*. On the other hand, the 2-ethyl-substituted alcohols give rise to α-ethyl fatty acids which are not readily oxidized *in vivo*. In fact, α-substituted fatty acids in which the substituent is larger than the methyl group are not readily oxidized in the body (for discussion see Williams (1947) and Breusch (1948)). This difference in the oxidation of the α-methyl and α-ethyl fatty acids is reflected in the glucuronic acid conjugation.

The main route of metabolism of the 2-methyl primary alcohols appears to be

Table 1. *Optical rotations of aliphatic ester glucuronides*

	Potassium salt [α] _D in water	Triacetyl methyl ester		
		M.p. (°)	[α] _D in CHCl ₃	[M] _D /100 in CHCl ₃
β -Glucuronide				
Acetyl*	—	172	+8.7	+32
α -Diethylacetyl	-27.1	72	0	0
α -Ethylhexanoyl	-27.5	82	0	0

* Synthetic (Goebel & Babers, 1934).

further oxidation products. There is some formation of the glucuronide, $\text{RCH}(\text{CH}_3)\text{CH}_2\text{O} \cdot \text{C}_6\text{H}_5\text{O}_6$. With the 2-ethyl alcohols, the main route of metabolism is

The product of oxidation of 2-ethylbutanol, however, is not completely resistant to oxidation, for a small amount of methyl *n*-propyl ketone was isolated from the urine. This ketone was also isolated by Blum & Koppel (1911) from the urine of a dog injected with diethylacetic acid, and presumably arises by decarboxylation of α -ethylacetoacetic acid, the β -oxidation product of diethylacetic acid. The metabolism of diethylethanol in the rabbit is thus mainly as follows:

2-Ethylhexanol gives rise to very large outputs of glucuronide, and nearly 90% of the dose can be accounted for as α -ethylhexanoylglucuronide. Its main route of metabolism in the rabbit is thus

According to Akiya & Yamakawa (1950) there is a further oxidation to a dibasic acid, and the present work suggests that a trace of ketone is also excreted. This ketone would be interesting if it could be isolated, since it could be either amyl methyl ketone or di-*n*-propyl ketone, or a mixture of both. Since 2-ethylhexanol contains an asymmetric carbon atom there is a possibility of resolution. The α -ethylhexanoic acid we isolated did in fact show optical activity corresponding to a mixture of 56% (–) isomer and 44% (+) isomer. At the moment, however, it cannot be decided whether the slight resolution had occurred biologically or chemically during isolation. Since 2-ethylhexanol gives very high outputs of ester glucuronide we believe that the resolution must have been the result of chemical manipulations. This point also raises the question of the nature of α -ethylhexanoylglucuronide, since the aglycone could be the (–) or (+) isomer or an equal or unequal mixture of these. The optical rotations of the known ester β -glucuronides of aliphatic acids are given in Table 1, the

acetyl derivative being synthetic (Goebel & Babers, 1934). The potassium salts of both diethylacetyl- and α -ethylhexanoylglucuronides have nearly the same rotation and this is also true of the triacetyl methyl esters. Since diethylacetic acid is optically inactive, the rotation of its glucuronide is that of an aliphatic ester glucuronide carrying an optically inactive aglycone. Since the glucuronide of α -ethylhexanoylglucuronide has approximately the same rotation, it is reasonable to conclude that here again the aglycone is inactive and that the glucuronide can be described as ($\pm$)- α -ethylhexanoyl- β -D-glucuronide, which is a partially racemic compound of the Ladenburg type. This is further supported by the fact that the triacetyl methyl ester has a sharp melting point.

Diethylacetyl- and α -ethylhexanoylglucuronides are the first ester glucuronides of fatty acids to be described. Ester glucuronides of the aromatic acids (e.g. benzoylglucuronide) are well known. It is noteworthy that these two aliphatic ester glucuronides appear to be much more stable than those of the aromatic series and only reduce Benedict's reagent relatively slowly.

SUMMARY

1. 2-Ethylbutanol is excreted by rabbits as an ester glucuronide which was proved by isolation to be diethylacetylglucuronide. A small amount of methyl *n*-propyl ketone is also excreted and this was isolated as the 2:4-dinitrophenylhydrazone.
2. Nearly 90% of DL-2-ethylhexanol is excreted by rabbits as α -ethylhexanoylglucuronide which was isolated and characterized as the potassium salt and triacetyl methyl ester.
3. The results are discussed and it is suggested that ester glucuronides may be formed from branched-chain fatty acids when β -oxidation is blocked.

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Studies on the Metabolism of Semen

8. ERGOTHIONEINE AS A NORMAL CONSTITUENT OF BOAR SEMINAL PLASMA. PURIFICATION AND CRYSTALLIZATION. SITE OF FORMATION AND FUNCTION

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The discovery of ergothioneine is due to Tanret (1909) who isolated it from ergot of rye and established its composition as $C_9H_{15}N_3O_2S$. Barger & Ewins (1911) identified the new substance as a betaine of thiolhistidine (β -2-thiolglyoxaline-4(5)-propionobetaine). They were also the first to point out that the sulphur of thiolglyoxaline is readily oxidized by ferric chloride or bromine to free sulphuric acid; this property, which distinguishes ergothioneine from cysteine and glutathione, has been utilized for the determination of ergothioneine (Blumenthal & Clarke, 1935; Touster, 1951). The final confirmation of the structure of ergothioneine was provided by Heath, Lawson & Rimington (1950, 1951) who succeeded in synthesizing ergothioneine from 2-thiolhistidine, an amino-acid which had been obtained synthetically (Ashley & Harington, 1930; Harington & Overhoff, 1933) but has hitherto never been found in nature and which is unable to replace histidine as a growth-promoting factor in animals (Neuberger & Webster, 1946).

Ergot, from which ergothioneine can be obtained in yields varying from 65 to 260 mg./100 g. (Eagles, 1928; Pirie, 1933a; Hunter, Molnar & Wight, 1949), remained the only known source of ergothioneine until Hunter & Eagles (1925, 1927) isolated from pig blood the crystalline substance X, later renamed sympectothion which, although different from uric acid, gave with phosphotungstic and arsenophosphotungstic acid reagents the same blue colour reaction as uric acid. A blood con-

stituent with similar properties, named thiasine, was isolated independently by Benedict, Newton & Behre (1926). Both sympectothion and thiasine were soon shown to be identical with ergothioneine (Newton, Benedict & Dakin, 1926; Eagles & Johnson, 1927). Blood ergothioneine, or 'thioneine' as it is sometimes called, is confined entirely to the erythrocytes and does not occur in the plasma. A sensitive and specific method for its estimation based on the diazo reaction has been developed by Hunter (1928, 1949). The content of ergothioneine in blood (mg./100 ml.) is much smaller than in ergot; it varies from 1.8 to 1.95 in man, 1.3 to 3.1 in rat, and 2.0 to 26.5 in pig (Hunter, 1951).

The present study reports the discovery of a third source of ergothioneine in nature, namely the seminal plasma (preliminary communication: Leone & Mann, 1951). Protein-free extracts from semen have been known for a long time to exhibit a marked reducing property towards 2:6-dichlorophenolindophenol, but this fact has usually been attributed to ascorbic acid (cf. Berg, Huggins & Hodges, 1941). We were able to confirm the occurrence of ascorbic acid in semen, but in addition we detected the presence of yet another reducing substance which has hitherto escaped recognition by other workers. This substance is ergothioneine. It occurs in a particularly high concentration in the semen of boar, and it originates in the seminal vesicles, the glands which also secrete fructose (Mann, 1946), citric acid (Humphrey & Mann, 1949) and inositol (Mann, 1951). In the secretion of the seminal vesicles, ergothioneine is present in a dialysable form. The

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