

Studies in Detoxication

14. THE METABOLISM OF VERATRALDEHYDE AND VERATRIC ACID IN THE RABBIT

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The work described in this paper had two objectives. First, it was hoped that a more stable aroylglucuronide (veratroylglucuronide and anisoylglucuronide) than benzoylglucuronide (Quick, 1926; Pryde & Williams, 1933; Goebel, 1938) could be prepared for constitutional studies. In the second place, it was considered possible that a comparative study of the metabolism of veratric acid (3:4-dimethoxybenzoic acid) and its aldehyde might throw light on the mode of oxidation of aromatic aldehydes to the corresponding acids, since early experiments had shown that the yields of crystalline veratroylglucuronide from rabbit urine were consistently higher when veratraldehyde was fed than when veratric acid was fed.

No previous study on the fate of veratraldehyde or of anisaldehyde has been recorded. Marfori (1897) states that veratric acid is excreted unchanged; our work will show this statement is only partly true.

METHODS AND RESULTS

Veratraldehyde (m.p. 46°) was prepared by the Reimer-Tiemann reaction (Johnson & Stevenson, 1936); veratric acid (m.p. 181°) was prepared in a similar manner (Kostanecki & Tambor, 1906). For feeding, the aldehyde was melted in warm water and the acid was neutralized with Na₂CO₃ solution.

Ethereal sulphate excretion. This was determined by the Folin gravimetric method (Williams, 1938). It was assumed that the increase in ethereal sulphate was due to conjugated catechol, 1 mol. of the phenol being combined with 1 or 2 of sulphuric acid. Hence in calculating the veratric aldehyde or acid equivalent to the ethereal sulphate increase, it was assumed that 1 or 2 mol. of SO₃ ≡ 1 mol. of veratric aldehyde or acid.

Total and free veratric acid excretion. The method used was identical with that of Sammons & Williams (1941) for the estimation of free and total vanillin

Table 1. *The distribution of metabolites in the urine of rabbits after the administration of veratric aldehyde and veratric acid*

(Urines for analysis were collected for 2 days after dosage.)

Substance fed	Rabbit no.	Dose (g.)	Free veratric acid		Total veratric acid		Veratroylglucuronide* % of dose	mg. SO ₃	Ethereal sulphate excretion calculated as		Total veratraldehyde or veratric acid excreted (% of dose)	
			(g.)	(% of dose)	(g.)	(% of dose)			Mono-sulphate (% of dose)	Disulphate (% of dose)	Including mono-sulphate	Including disulphate
Veratraldehyde	71	2.2	0.790	35.7	1.440	64.9	29.2	75	7.0	3.5	71.9	68.4
	76	2.2	0.261	10.8	1.462	60.6	49.8	48	4.4	2.2	65.0	62.8
	77	1.9	0.503	24.2	1.348	64.7	40.5	50	5.2	2.6	69.9	67.3
	78	2.2	0.983	40.8	1.500	62.2	21.4	43	4.0	2.0	66.2	64.2
	78	2.2	0.318	13.2	1.564	64.8	51.6	68	6.2	3.1	71.0	67.9
	79	1.9	0.848	40.7	1.641	78.7	38.0	64	6.6	3.3	85.3	82.0
Average				(27.6)		66.0	(38.4)		5.6	2.8	71.6	68.8
Veratric acid	76	2.0	0.750	37.4	1.390	69.5	32.0	58	5.8	2.9	75.3	72.4
	77	1.9	0.725	38.1	1.290	67.9	29.8	42	4.4	2.2	72.3	70.1
	78	2.25	0.897	39.9	1.579	70.2	30.3	25	2.2	1.1	72.4	71.3
	79	2.0	0.984	49.2	1.417	70.8	21.6	41	4.0	2.0	74.8	72.8
Average				41.2		69.6	28.4		4.2	2.1	75.8	71.6

* I.e. difference between excreted total and free veratric acid.

acid in urine, i.e. ether extraction and a methoxyl estimation. The method was found to be reliable for total veratric acid, but the amount of free veratric acid was uncertain owing to the instability of veratroylglucuronide in urine. The results are given in Table 1.

Isolation of veratroylglucuronide

(a) *From veratraldehyde.* 2 g. of veratraldehyde were administered by stomach tube to each of ten rabbits and the urine (1620 ml.) collected during the following 24 hr. All reagents were ice-cold and were stored at 0°. The urine, acidified with a few drops of glacial acetic acid, was treated with 400 ml. of saturated normal lead acetate and centrifuged. The precipitate was discarded and the clear supernatant liquor made faintly alkaline with a few drops of strong ammonia. Saturated basic lead acetate (500 ml.) was now added and the whole centrifuged. The precipitate was washed twice on the centrifuge with ice-cold water and made into a thin cream with water. The lead was removed with H₂S and the filtrate aerated. *Veratroylglucuronide* crystallized overnight at 0°. A further quantity was obtained on concentrating the mother liquor *in vacuo* at 30–35° (total yield, 11 g.). On recrystallization from hot water, it formed needles, m.p. 169–170°, showing $[\alpha]_D^{23} - 13.1^\circ$ ($c = 0.9$ in water) and $[\alpha]_D^{24} - 2.5^\circ$ ($c = 1$ in methanol). From methanol the glucuronide separated as a gelatinous mass which crystallized only when water was added. It reduced Benedict's and Fehling's solution and was soluble in methanol, acetone and methylethylketone, sparingly soluble in ethyl acetate and cold water and insoluble in ether. (Found: C, 45.9; H, 5.8; OMe, 15.9; H₂O, 8.8 %; equiv., 393, after hydrolysis, 195. C₁₅H₁₈O₁₀·2H₂O requires C, 45.7; H, 5.6; OMe, 15.7; H₂O, 9.1 %; equiv., 394, after hydrolysis 197.)

(b) *From veratric acid.* A rabbit was given 2 g. veratric acid dissolved in dilute Na₂CO₃. The procedure was the same as described in the preceding paragraph and 0.5 g. of veratroylglucuronide was isolated, m.p. 169° after recrystallization. (Found: H₂O, 8.9 %. Calc. for 2H₂O, 9.1 %.)

The action of NaCN on veratroylglucuronide

Quick (1926; cf. Pryde & Williams, 1933) showed that when benzoylglucuronide was treated with NaCN, it underwent a change in optical rotation, for which there is no complete explanation. Goebel (1938) suggests that the phenomenon is the result of a complex series of reactions initiated by a migration of the benzoyl group, catalyzed by OH ions, from the aldehydic to one of the remaining carbon atoms of glucuronic acid. The following experiments show that veratroylglucuronide undergoes a similar rotatory change. On treatment of a 1 % solution of the glucuronide with 1.5 molecular equivalents

of NaCN, the rotation of the solution changed from $[\alpha]_D^{23} - 13.4^\circ$ to $+1^\circ$ in 110 min. With increase of the amount of NaCN to 2 mol., the change was less rapid, $[\alpha]_D^{24} - 15.8^\circ$ to -7.1° in 6.5 hr. and similar results were observed with 1 or 5 % solutions of the glucuronide. When the cyanide-treated solution had reached a constant rotation, it was no longer reducing to Benedict's or Fehling's solution.

1 g. of the glucuronide (1 mol.) and 0.2487 g. of NaCN (2 mol.) were dissolved in 20 ml. water. When the solution had attained a constant rotation (30 hr.) it was acidified with 2 ml. of 2N-HCl and then extracted with ether. Removal of the ether left 0.156 g. (37.5 % theory) of veratric acid, m.p. and mixed m.p. 179°, after recrystallization. The solution which remained after ether extraction was made slightly acid to congo-red and then taken to a syrup which was non-reducing. No positive result concerning the identity of this syrup was obtained.

Veratroylglucuronide methyl ester

This was prepared in three ways (diazomethane, methyl alcoholic HCl and methyl iodide plus silver oxide in the cold) and each method gave the same product. Only one method need be described here. Anhydrous veratroylglucuronide (2.7 g.) was dissolved in dry methanol (25 ml.) by heating under reflux. The solution was cooled to -5° and treated with diazomethane in the usual manner. After 2 hr. at 0°, the solution was concentrated at 35° to a gelatinous solid which was purified by precipitation from methanol with ether. The *ester* had m.p. 167° and $[\alpha]_D^{26} - 9.9^\circ$ ($c = 1$ in water). It reduced Fehling's and Benedict's reagents and gave a bright yellow colour with NaOH on standing or warming. (Found: OMe, 21 %. C₁₆H₂₀O₁₀ requires OMe, 25.0 %.)

This methyl ester (1 g.) was now dissolved in pyridine (10 ml.) and acetic anhydride (6 ml.) and allowed to stand overnight. The solution was then poured into water and the white crystalline precipitate (0.5 g.) was collected and recrystallized from aqueous methanol. The *triacetylveratroylglucuronide methyl ester* formed large needles, m.p. 144.5° and $[\alpha]_D^{24} - 31.3^\circ$ ($c = 0.9$ in methanol) and was reducing. (Found: C, 53.2; H, 5.5; OMe, 18.2 %. C₂₃H₂₆O₁₃ requires C, 53.0; H, 5.3; OMe, 18.7 %.)

Complete methylation of the methyl ester. Several attempts were made to methylate veratroylglucuronide with methyl iodide and silver oxide. The product obtained was a pale yellow syrup, slightly acidic to congo-red paper and reducing to Benedict's reagent. The methoxyl content was 41.5 %; fully methylated veratroylglucuronide requires 44.9 %. This product was not homogeneous and could be fractionated by means of light petroleum and ether mixtures. Although much time was spent (see Sammons, 1942) in investigating the nature of this syrup, the only definite hydrolytic product

was veratric acid. The results indicated that veratroylglucuronide was not stable during methylation, although the veratroyl group was not removed during the process.

*Hydrolysis of veratroylglucuronide
by normal rabbit urine*

Veratroylglucuronide was dissolved in normal rabbit urine (alkaline) and the free veratric acid content was determined at intervals. The results for the urine of two different rabbits are given in Table 2.

Table 2. *The decomposition of veratroylglucuronide in rabbit urine*

Vol. of urine (ml.)	Glucuronide added (g.)	Glucuronide hydrolyzed (%) in (hr.)			
		0	2	24	48
50	0.43	—	17.9	26.5	83.1
50	0.16	9.7	—	85.6	—

From these urines, crystalline veratric acid, identical with an authentic sample, was isolated by extraction with ether.

Isolation of free veratric acid from veratraldehyde urine

A rabbit was given 2 g. of the aldehyde and the urine (90 ml.) collected for 24 hr. Sulphuric acid (9 ml. of 10 %) was added to the urine, which was then extracted with ether for 4 hr. Evaporation of the ether gave a semi-crystalline residue which was now twice extracted with hot toluene. Veratric acid (0.26 g. or 12 % of the dose fed, m.p. and mixed m.p. 181° after recrystallization from water) was obtained on evaporation of the toluene extract. The residue after extraction with toluene was tested for vanillic acid by the colour reaction of Thorpe & Williams (1937) with uniformly negative results. No evidence was obtained for the presence in this residue of veratroylglycine, although if a glycine derivative were formed it would be expected to occur here.

Examination of the urine for free veratraldehyde

Urine from rabbits fed with veratraldehyde (1 g./kg.) was collected periodically (2, 4 and 24 hr. after feeding). All urines gave negative tests for veratraldehyde. The tests used were the colour reaction of Sammons & Williams (1941) for *p*-methoxybenzaldehydes and the use of 2:4-dinitrophenylhydrazine. No unchanged aldehyde group was detected although free and conjugated veratric acid was being excreted 4 hr. after feeding the aldehyde.

Examination of the urine for catechol and protocatechuic acid

After the administration of either veratraldehyde or veratric acid the urine gave a green colour (red in alkali) in neutral solution with FeCl_3 after acid hydrolysis. No reaction was obtained with unhydrolyzed urines. This indicated that the urines contained either catechol or protocatechuic acid or both. Both these substances are possible metabolic products of veratraldehyde. Preusse (1878-9) has devised a method whereby catechol and protocatechuic acid can be detected in the presence of each other in biological material such as urine. The principle of the method is that whereas both catechol and protocatechuic acid can be extracted by ether from acid solutions, only catechol can be extracted from alkaline (Na_2CO_3) solutions. 50 ml. of 'veratraldehyde urine' were made alkaline with Na_2CO_3 and extracted in a continuous extractor with ether. The ether was evaporated and the residue dissolved in water. This solution gave a green colour with FeCl_3 indicating the presence of free catechol. The alkaline urine was now acidified and re-extracted with ether. The residue from the ether extract gave no colour with FeCl_3 showing that no free protocatechuic acid was present. The acidified urine, thus twice extracted, was now boiled to hydrolyze conjugated compounds and again extracted with ether when alkaline and acid. The alkaline but not the acid extract again gave a green colour with FeCl_3 , thus indicating the presence of conjugated catechol but not of conjugated protocatechuic acid. Similar, but much more striking results were obtained on the filtrate from veratroylglucuronide as obtained from the basic lead acetate precipitate above.

Isolation of tetrabromocatechol. The basic lead precipitate from rabbit urine after veratraldehyde feeding was treated with H_2S . The PbS was filtered off and the veratroylglucuronide isolated as described above. The mother liquors from the preparation of the glucuronide were made alkaline with Na_2CO_3 and continuously extracted with ether. The extract (a) contained catechol. The solution was now made acid and extracted (b) but no colour was obtained with FeCl_3 . The acid solution was now boiled for an hour, made alkaline by Na_2CO_3 and extracted with ether. The extract (c) contained catechol. On acidification and further extraction, nothing which reacted with FeCl_3 was found. The ether extracts (a) and (c) were evaporated to a reddish brown syrup. Attempts to sublime the catechol from the syrup were not successful. The syrup was therefore extracted with CCl_4 and the extract treated with a few drops of bromine. On standing overnight yellowish needles (50 mg.) separated. These were collected and twice recrystallized

from methanol containing a little water. Finally, 15 mg. of slightly coloured tetrabromocatechol were obtained. They gave a dark blue colour with FeCl_3 and had m.p. 188° which was not depressed by an authentic sample, m.p. 192° . (Found: Br, 70.3 %. Calc. for $\text{C}_6\text{H}_2\text{O}_2\text{Br}_4$: Br, 75.1 %.) Stenhouse (1875) gives m.p. 187° and Frejka & Sefránek (1935) give m.p. 191 – 192° .

Anisoylglucuronide

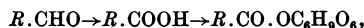
2 g. of anisaldehyde (*p*-methoxybenzaldehyde, b.p. 248°) were fed to each of six rabbits. The urine was collected for 24 hr. and the procedure for the isolation of veratroylglucuronide followed exactly. *Anisoylglucuronide* was obtained as a non-crystalline gelatinous mass which dried to an amorphous white powder (yield, 1.5 g.). It could not be crystallized from any solvent tried. It was acidic and laevorotatory; it reduced Benedict's reagent and gave an intense naphthoresorcinol reaction.

Triacetylanisoylglucuronide methyl ester. Dry anisoylglucuronide (0.5 g.) was converted to an amorphous *anisoylglucuronide methyl ester* with methanol saturated with gaseous HCl at 0° . The ester (0.4 g.) was acetylated as described for the veratroyl derivative. *Triacetylanisoylglucuronide methyl ester* (0.4 g.) was isolated as large needles (from aqueous methanol), m.p. 158° and $[\alpha]_D^{21} = -20.5^\circ$ ($c = 1.2$ in chloroform). It was not sufficiently soluble in methanol to give a 1 % solution. (Found: C, 53.9; H, 5.3; OMe, 13.5 %. $\text{C}_{21}\text{H}_{25}\text{O}_{12}$ requires C, 53.8; H, 5.2; OMe, 13.2 %.)

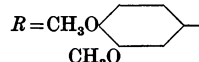
DISCUSSION

Approximately 70 % of the veratric acid and aldehyde fed to rabbits can be accounted for as metabolites excreted in the urine. The main metabolites in each case are veratric acid and veratroylglucuronide. The relative amounts of these metabolites excreted, however, are uncertain, for, whereas the estimations of total veratric acid excreted are reliable, those of free veratric acid and consequently of veratroylglucuronide are unreliable (see Table 1). The reason for this became clear when a study of the stability of veratroylglucuronide in normal rabbit urine was carried out. This compound decomposes fairly rapidly in rabbit urine (Table 2), presumably owing to the alkalinity of the latter. The figures in Table 1 do suggest, however, that the excretion of veratroylglucuronide is greater after feeding the aldehyde than after the acid. This suggestion receives some support from the observation that the yields of the crystalline glucuronide were always greater when the aldehyde was fed than when the acid was fed. But we feel that our results do not allow us to make a decision on this point.

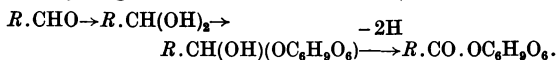
If it were true that the glucuronide output from the aldehyde was greater than from the acid, then the possibility arises that excreted veratroylglucuronide is not formed from veratraldehyde only via veratric acid thus:



where

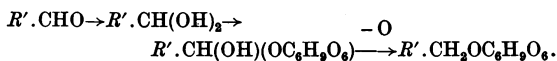


An alternative possibility is that the intermediates are the aldehyde hydrate and its glucuronide, the latter being converted to veratroylglucuronide by a dehydrogenation thus:



The notion that an aldehyde may be oxidized, chemically and biologically, to an acid via the aldehyde hydrate is well known (cf. Wieland, 1932).

A similar possibility arises when aldehydes are converted to the glucuronides of the corresponding alcohols thus:



This latter suggestion receives some support from the work of Lehmann & Knoefel (1938) on the pharmacodynamics of chloral hydrate and trichloroethanol. It is generally believed that chloral hydrate is detoxicated by being reduced to trichloroethanol, which then conjugates to form trichloroethylglucuronide (urochloralic acid). Lehmann & Knoefel (1938) found that the duration of the hypnotic action of chloral hydrate is briefer than that of trichloroethanol and that the conversion of the latter to inactive metabolites proceeds very slowly. They further state that 'if chloral hydrate were reduced in the organism to trichloroethanol, the duration of action of the former should be longer, as an extra step is required for its detoxication. Since the effect of chloral hydrate is briefer, it seems unlikely that it forms trichloroethanol as such'.

It is, therefore, obvious that there may be some doubt about the mechanism of the transformation of aldehydes to the glucuronides of the corresponding acids or alcohols, and further elucidation is necessary.

The possibility of the formation of veratroylglucuronide from the aldehyde via the acid, however, cannot be entirely eliminated, since the acid itself forms the glucuronide. This process (with the aldehyde hydrate as an intermediate) could be represented as follows:



Catechol is also a metabolite of both veratric aldehyde and acid. Its formation from veratraldehyde involves oxidation, decarboxylation and de-

methylation. It appears in the urine in a combined form which could be a di- or a mono-etheral sulphate (cf. adrenaline; Richter, 1940; Deichmann, 1943).

The preparation of anisoylglucuronide has not been described before, but Quick (1932) has reported that the corresponding acid, anisic acid, gives rise in the dog and in man to a glucuronide which was not isolated and to a glycine conjugate, *p*-methoxyhippuric acid, which was isolated.

SUMMARY

The fate of veratraldehyde of veratric acid and of anisaldehyde in the rabbit has been studied:

(1) In the case of veratraldehyde, about 70 % of the dose can be accounted for as veratric acid (c. 28 %) and veratroylglucuronide (c. 38 %). There is some uncertainty regarding the relative amounts of these two metabolites owing to the instability of the glucuronide in rabbit urine.

(2) About 70 % of veratric acid can also be accounted for, about 41 % as free veratric acid and about 28 % as its glucuronide.

(3) The results suggest that the amount of glucuronide formed from the aldehyde is greater than from the acid. The implications of this suggestion are discussed.

(4) A minor metabolite of both veratric acid and aldehyde was identified as catechol, which was responsible for the small increase in etheral sulphate output. Vanillic acid derivatives, veratroylglycine and protocatechuic acid were not found in the urine.

(5) Veratroylglucuronide and some of its derivatives have been prepared and some of their properties described.

(6) Anisoylglucuronide has been prepared from the urine of anisaldehyde-fed rabbits and some of its derivatives have been described.

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The Esterification of Glycerol with Hippuric Acid and the Behaviour of the Resulting Product towards Enzymes

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The present paper is a report on the first of a series of ester compounds of glycerol with amino-acid derivatives which are being synthesized in order to investigate their chemical properties and behaviour towards enzymes. The synthesis of esters of the amino-acids with glycerol has been twice reported. Abderhalden & Guggenheim (1910) obtained the ester of tyrosine with glycerol and later Fodor & Weizmann (1926) and Weizmann, Haskelberg & Malkova (1929) (cf. Haskelberg, 1930) prepared the mono-ester of glycerol with leucine. Though other esters of glycerol with amino-acids (alanine and glycine esters) were prepared by

Fodor & Weizmann (1910) crystallization of these compounds presented great difficulty and in the case of the alanine and glycine esters was unsuccessful.

This paper describes an attempt to obtain a crystalline product from glycerol and glycine by first substituting the free amino group of glycine with the benzoyl radical as in hippuric acid. The behaviour of the resulting ester towards lipases and esterases was investigated, but this could not be done in the case of the leucine ester owing to its extreme instability in aqueous solution. Preliminary work on the esters of glutamic acid and