Studies in Detoxication

57. THE STRUCTURE OF THE GLUCURONIDES FROM 2- AND 4-QUINOLONES AND THE ORIENTATION OF BIOLOGICAL HYDROXYLATION IN QUINOLONES

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Fenyvessy (1900) fed carbostyril (2-quinolone or 2-hydroxyquinoline) to rabbits, and isolated from the urine a carbostyril glucuronide (otherwise known as a carbostyril glucosiduronic acid, see paper 55, p. 317), which was regarded as quinolyl-2-glucuronide. Smith (1953), however, showed that this compound was a derivative of a hydroxy-2-quinolone in which glucuronic acid was attached to the quinoline ring elsewhere than in the 2-position. Fenyvessy (1900) also showed that 4-quinolone yielded a glucuronide, but it was not characterized. Smith (1953), however, showed that 4-quinolone (4-hydroxyquinoline) gave rise to two glucuronides in the rabbit. One was crystalline, and the glucuronic acid was attached to the quinoline ring at a position other than the 4-position. The second glucuronide was amorphous and was not fully described. In the present paper, evidence will be presented to show that Fenyvessy's carbostyril glucuronide (Smith's 2-quinolonyl-*x*-glucuronide) is 2-quinolonyl-6-glucuronide, and that the two glucuronides from 4-quinolone are 4-quinolonyl-3- and 4-quinolonyl-6-glucuronide.

EXPERIMENTAL

Melting points are uncorrected.

Reference compounds

2-Quinolone (carbostyril), m.p. 200°, was purchased (The British Drug Houses Ltd., London). The following were prepared: 4-quinolone, m.p. 200° (Albert & Magrath, 1947); 7-hydroxy-2-quinolone, m.p. 310° (decomp.) (Koelsch & Walker, 1950) and 6-hydroxy-2-quinolone (Gattermann, 1894). 6-Hydroxy-2-quinolone was acetylated with pyridine and acetic anhydride. The product was poured into water, giving a crystalline precipitate, which on repeated recrystallization from aqueous ethanol yielded 6-*acetoxy*-2-*quinolone* as colourless plates, m.p. 225–226° (decomp.). (Found: C, 64·8; H, 4·6; N, 6·4. $C_{11}H_9O_3N$ requires C, 65·0; H, 4·5; N, 6·9%.)

6-Hydroxy-4-quinolone was prepared by demethylation of the 6-methoxy derivative. The latter (2·5 g.), prepared according to Riegel, Albisetti, Lappin & Baker (1946), was boiled in conc. HBr (50 ml.) for 4 hr. On cooling, 2·7 g. of 6-hydroxy-4-quinolone hydrobromide separated. The hydrobromide was dissolved in water and the free phenol precipitated by adding $NaHCO_3$. 6-Hydroxy-4-quinolone has been previously described by Hirsch (1896) and has no definite melting point.

Identification of the glucuronides

The glucuronide of carbostyril. Smith (1953) designated this glucuronide '2-quinolonyl-*x*-glucuronide', where *x* could be 3, 4, 5, 6, 7, or 8. A little of the glucuronide was hydrolysed, and a small amount of the phenol isolated, as described below, for preliminary colour tests. The phenol did not reduce ammoniacal silver nitrate, showing that it was unlikely to be 2:3-dihydroxyquinoline. With aqueous $FeCl_3$, it gave a weak, yellow-brown colour, which seemed to eliminate 2:3- and 2:8-dihydroxyquinoline. 2:3-Dihydroxyquinoline gives a greenish blue-black colour with $FeCl_3$ (Madelung, 1912), and the 2:8-derivative a dirty green colour, since it is a derivative of 8-hydroxyquinoline (Diamant, 1895). 2:4-Dihydroxyquinoline in an ammoniacal solution turns blue when shaken in the presence of air or H_2O_2 (Ashley, Perkin & Robinson, 1930), but the above phenol did not give this test. 2:5-Dihydroxyquinoline would be expected to give a colour with 2:6-dichloroquinone chloroimide, since position 8, which is *para* to position 5, is unsubstituted (cf. Gibbs, 1927); the above phenol, however, gave no colour with this reagent. The phenol was thus probably 2:6- or 2:7-dihydroxyquinoline, which are known compounds (see above). These two compounds could be distinguished chromatographically, spectrographically, and by their fluorescence in ultraviolet light (see Table 1).

Carbostyril glucuronide (m.p. 250–252°; 1·3 g.) (cf. Smith, 1953) was heated under reflux in 50 ml. 2N-HCl for 4 hr. The solution was then evaporated on a water bath to a small volume, treated with charcoal, and filtered. On cooling, 6-*hydroxy*-2-*quinolone hydrochloride* separated as colourless needles which were recrystallized from 2N-HCl (yield, 0·15 g.). (Found: C, 50·4; H, 4·7; N, 6·3. $C_9H_8O_2NCl$, H_2O requires C, 50·1; H, 4·6; N, 6·5%.) The spectra of this hydrochloride in 0·1N-HCl and 0·1N-NaOH were identical with those of 6-hydroxy-2-quinolone in the same solvents (see Fig. 1).

This hydrochloride (20 mg.) was dissolved in a little acetic anhydride containing a trace of pyridine, the solution was raised to the boiling point and then cooled. On pouring the product into water, crystals, m.p. 221–223°, separated which when analysed were recognized as 2:6-*dihydroxyquinoline diacetate.* (Found: C, 63·7; H, 4·5. $C_{13}H_{11}O_4N$ requires C, 63·3; H, 4·4%.) On repeated recrystallization of this compound from aqueous ethanol, 6-acetoxy-2-quinolone, m.p. and mixed m.p. 223–228°, identical with the authentic compound was obtained. (Found: C, 65·3; H, 4·65; N, 7·1. Calc. for $C_{11}H_9O_3N$: C, 65·0; H, 4·5; N, 6·9%.)

Table 1. *Properties of* 2:6-, 2:7- *and* 3:4-*dihydroxyquinolines*

Position of substitution in dihydroxy quinoline	Colour of fluorescence in u.v. light*		R_F values†			Spectra			
						In 0·1 N-HCl		In 0·1 N-NaOH	
	In ammonia	In HCl	In benzene: *n*-butanol: ammonia (sp.gr. 0·88), 2:5:2, v/v	In *n*-butanol: acetic acid: water, 4:1:5, v/v	In *n*-butanol saturated with water	$\lambda_{max.}$ mμ.	$\epsilon_{max.}$	$\lambda_{max.}$ mμ.	$\epsilon_{max.}$
2:7	Blue	Weak blue	0·25	0·8	Not measured	250 323	2 200 5 100	267 347	2 700 7 600
2:6	Green	Blue	0·6	0·8	0·8	230 350	31 700 5 100	245 380	34 000 4 800
2:*x* (metabolic product)	Green	Blue	0·6	0·8	0·8	230 350	31 100 5 000	245 380	34 200 5 000
3:4	Blue	Blue	—‡	0·95	0·8	234 332	56 000 6 800	—‡	—‡
3:4 (metabolic product)	Blue	Blue	—‡	0·95	0·8	235 332	56 600 6 700	—‡	—‡

* A drop of an aqueous solution of the dihydroxyquinolines was spotted on filter paper and allowed to dry. The spot was examined under u.v. light after treatment with NH_3 or HCl fumes.

† Chromatograms run until front had moved 12 in. on Whatman nos. 1 or 4 paper.

‡ 3:4-Dihydroxyquinoline decomposes in alkaline solutions.

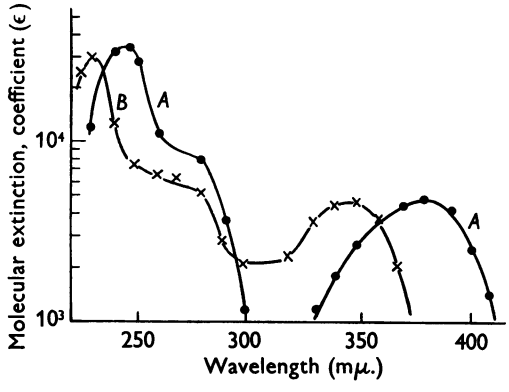


Fig. 1. Absorption spectrum of authentic 2:6-dihydroxyquinoline, *A* (full line) in 0·1 N-NaOH and *B* (full line) in 0·1 N-HCl; and of 2-*x*-dihydroxyquinoline, ●—●, in 0·1 N-NaOH, and ×—×, in 0·1 N-HCl.

Glucuronides isolated after feeding with 4-*quinolone.* (1) *Identification of the crystalline glucuronide, m.p.* 208°. Smith (1953) isolated in small yield a crystalline glucuronide from the urine of rabbits fed with 4-quinolone. This glucuronide, which was a trihydrate, had m.p. 208° (decomp.), $[\alpha]_D^{20} - 128°$ in water (*c*, 0·015). It formed a sodium salt, $[\alpha]_D^{20} - 111°$ in water (*c*, 1). The glucuronide and its sodium salt have now been prepared biosynthetically by feeding 6-hydroxy-4-quinoline to rabbits and the '4-quinolonyl-*x*-glucuronide' of Smith (1953) has thus been identified as 4-quinolonyl-6-β-D-glucosiduronic acid.

The phenol obtained by hydrolysis of the glucuronide gave a red colour with $FeCl_3$ (cf. Hirsch, 1896) and decomposed at about 280°. This suggested that our compound was 4:6-dihydroxyquinoline, or possibly the unknown 4:7-derivative. The 2:4-, 3:4- and 4:8-dihydroxyquinolines give different colour reactions (see above), and the 4:5-derivative melts at 231° (Musajo & Minchilli, 1941).

6-Hydroxy-4-quinolone (1 g.) was therefore fed in water by stomach tube to a rabbit (2·5 kg.). The 24 hr. urine was collected and the glucuronide isolated as the basic lead salt (cf. Kamil, Smith & Williams, 1951). The lead salt was suspended in water (150 ml.) and H_2S passed through the suspension until all the lead had been converted into PbS. The mixture was then boiled and filtered hot to remove PbS. On cooling, 4-quinolonyl-6-β-D-glucosiduronic acid separated as fine, colourless needles (0·3 g.). After recrystallization from water, the glucuronide was obtained as a trihydrate, m.p. and mixed m.p. with 4-quinolonyl-*x*-glucuronide, 208° (decomp.) and $[\alpha]_D - 122°$ in water (*c*, 0·015). (Found: C, 45·9; H, 5·5; N, 3·7. Calc. for $C_{15}H_{15}O_8N$, $3H_2O$: C, 46·0; H, 5·4; N, 3·6%.) The glucuronide gave a strong naphthoresorcinol reaction and was very sparingly soluble in water. On treatment of the glucuronide (50 mg.) in a little water with one equivalent of $NaHCO_3$ (12 mg.), a solution of the sodium salt was obtained. On addition of ethanol, the sodium salt separated, $[\alpha]_D^{20} - 110°$ in water (*c*, 1). (Found: N, 3·4; Na, 5·8. Calc. for $C_{15}H_{14}O_8NNa$, 3·5 H_2O: N, 3·3; Na, 5·4%.)

The above glucuronide and its sodium salt were identical in all respects with the 4-quinolonyl-*x*-glucuronide, and its sodium salt. The identical ultraviolet absorption spectra in acid and alkali of the two glucuronides are shown in Fig. 2.

(2) *Identification of a second glucuronide.* Smith (1953) showed that, after sodium 4-quinolonyl-6-glucosiduronate had been separated from the glucuronide fraction of 4-quinolone urine, another glucuronide could, by the addition of ethanol, be isolated in good yield as an amorphous sodium salt. This glucuronide has now been characterized as a toluidine salt.

The crude glucuronide gum (1·5 g.) from the 24 hr. urine of three rabbits which had each received 1·5 g. of 4-quinolone was isolated by the lead acetate procedure. The gum was dissolved in water (10 ml.) and the glucuronide precipitated by adding acetone (50 ml.). The filtrate from the glucuronide contained ethereal sulphate. The glucuronide precipitate still contained some inorganic material which was removed by stirring the glucuronide for 0·5 hr. with 10 g. of Amberlite I.R. 100 (H) resin in 20 ml. water. The solution was decanted and evaporated to dryness *in vacuo* to a buff residue (0·5 g.). A portion of this material (0·3 g.) was mixed with *p*-toluidine (0·1 g.) and warmed with 2 ml. water. After 24 hr., the crystalline *p-toluidine salt* of *4-quinolonyl-3-glucosiduronic acid* (0·15 g.) was collected and washed with water, ethanol and, finally, ether. On recrystallization from water, it formed colourless needles, m.p. 174–177° (decomp.), $[\alpha]_D^{25}$ – 108° in water (*c*, 0·5). (Found: C, 56·6; H, 5·3; N, 6·5. $C_{22}H_{24}O_8N_2$, H_2O requires C, 57·1; H, 5·7; N, 6·1%.) It was sparingly soluble in cold water, but more soluble in aqueous ethanol. After exposure to ammonia fumes, a spot of this compound on filter paper gave a purple fluorescence in ultraviolet light; this fluorescence disappeared when the spot was exposed to HCl fumes. The R_F value of the glucuronide was 0·5 in *n*-butanol: acetic acid: water (4:1:5, v/v) and 0·2 in benzene: *n*-butanol: ammonia (sp.gr. 0·880) (2:5:2, v/v). The glucuronide was relatively resistant to acid hydrolysis and gave a positive result with the naphthoresorcinol test only after prolonged boiling.

The glucuronide did not yield 4-quinolone on hydrolysis, but a phenol which gave a green colour with $FeCl_3$ in weakly acid solution, suggesting that it was 3:4- or 4:8-dihydroxyquinoline. On adding $NaHCO_3$, the colour changed to blue and then to red, which is characteristic of 3:4-dihydroxyquinoline (Coppini, 1950). The phenol also reduced ammoniacal $AgNO_3$, thus confirming its identification as the 3:4-derivative.

The amorphous sodium salt (1·2 g.) was dissolved in 50 ml. 2 N-HCl and boiled for 4 hr. under reflux. The solution was then concentrated on a water bath to a small volume and crystals of the supposed *3-hydroxy-4-quinolone hydrochloride* separated (130 mg.). On recrystallization from 2 N-HCl, the hydrochloride formed colourless needles having no definite m.p. (Found: N, 7·0. $C_9H_7O_2N$, HCl requires N, 7·1%.) The free 3-hydroxy-4-quinolone was liberated as an insoluble precipitate by treatment of an aqueous solution with $NaHCO_3$ and purified by sublimation *in vacuo* and recrystallization from methanol-dioxan. It had m.p. 245–250° (decomp.) and did not depress the m.p. of an authentic sample of 3-hydroxy-4-quinolone (m.p. 245–250°, decomp.) kindly provided by Dr D. Coppini (cf. Coppini, 1950). In his paper, Coppini states that this compound melts between 280 and 315°, depending on the rate of heating, but the few mg. he sent us melted as quoted. However, the decomposition points of compounds in this series are too high to be reliable. The spectra of the metabolic and authentic samples were identical (see Fig. 3) and they showed identical behaviour on paper chromatograms (see Table 1). On benzoylation of the above dihydroxyquinoline with benzoyl chloride and NaOH, 3:4-dihydroxyquinoline dibenzoate, m.p. 129°, was obtained. (Found: C, 74·8; H, 4·2; N, 4·3. Calc. for $C_{23}H_{15}O_4N$; C, 74·8; H, 4·1; N, 3·8%.) Coppini (1950) quotes m.p. 123–124° for this dibenzoate.

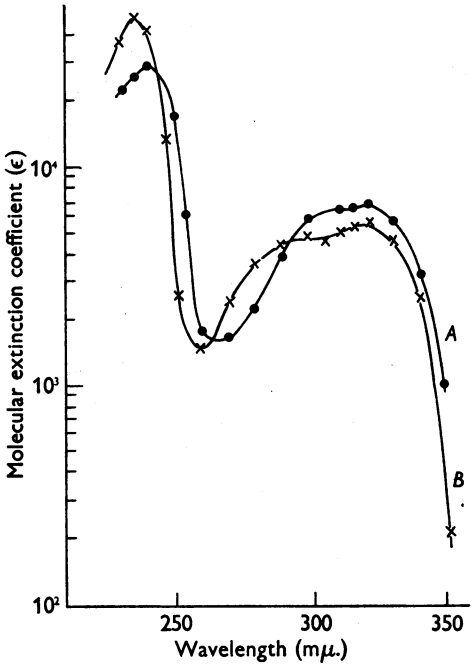


Fig. 2. Absorption spectrum of biosynthesized 4-quinolonyl-6-glucosiduronic acid obtained by feeding with 6-hydroxy-4-quinolone, *A* (full line) in 0·1 N-NaOH and *B* (full line) in 0·1 N-HCl and of '4-quinolonyl-*x*-glucuronide' obtained by feeding with 4-quinolone, ●—●, in 0·1 N-NaOH, and × — ×, in 0·1 N-HCl. For the biosynthesized glucuronide in 0·1 N-NaOH, $\lambda_{max.}$ = 240, 310, 320 mμ. with $\epsilon_{max.}$ = 30 000, 6400, 6600 and in 0·1 N-HCl $\lambda_{max.}$ = 235, 299, 320 mμ. with $\epsilon_{max.}$ = 52 500, 4800, 5400.

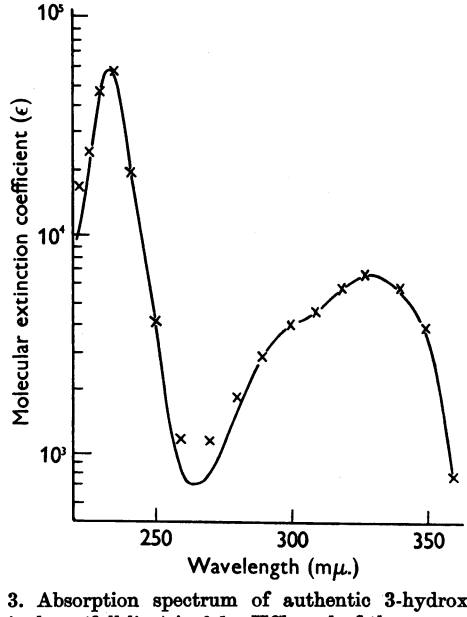
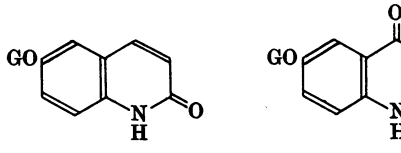


Fig. 3. Absorption spectrum of authentic 3-hydroxy-4-quinolone (full line) in 0·1 N-HCl, and of the same compound obtained by feeding with 4-quinolone (× — ×).

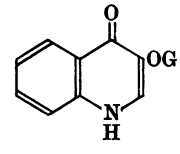
DISCUSSION

Smith (1953) concluded from spectroscopic data, that the glucuronide (I), m.p. 250–252°, obtained by feeding with 2-quinolone, and the crystalline glucuronide (II), m.p. 208°, obtained by feeding with 4-quinolone, were derivatives of hydroxyquinolones, and the present work shows them to be 6-hydroxy derivatives. The second glucuronide (*p*-toluidine salt, m.p. 174–177°) obtained by feeding with 4-quinolone might have been

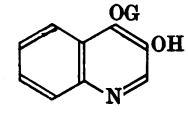


I

II



III



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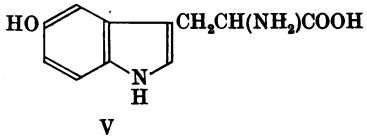

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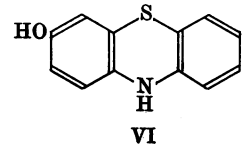

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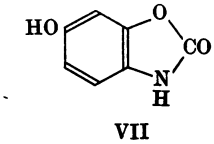

VII

4-quinolonyl-3-glucosiduronic acid (III) or 3-hydroxyquinolyl-4-glucosiduronic acid (IV). By analogy with I and II, it would be expected to be III. The ultraviolet absorption spectra of this glucuronide in acid and alkali are given in Fig. 4. The shift in the long-wave maximum at 325 mμ. on moving from acid to alkali is very small. It has been shown by Steck, Ewing & Nachod (1949) that this is characteristic of the quinolone structure, thus supporting structure III. This glucuronide is also highly resistant to acid hydrolysis. If the compound had structure IV, it would be expected to be easily hydrolysed, since it is known that 2- and 4-alkoxyquinolines are hydrolysed with great ease by dilute hydrochloric acid, whereas the aromatic quinolyl ethers are much more stable (see Elderfield, 1952). We therefore conclude that the glucuronide yielding 3:4-dihydroxyquinoline is III.

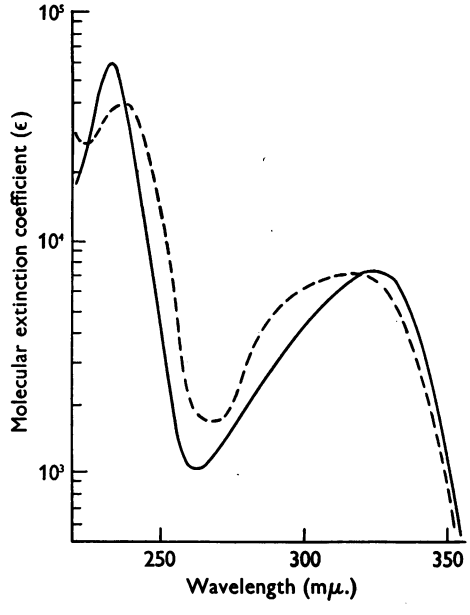

Fig. 4. Absorption spectrum of *p*-toluidine salt of 4-quinolonyl-3-glucosiduronic acid in 0·1 N-NaOH (broken line) and in 0·1 N-HCl (full line). In NaOH, $\lambda_{max.} = 238$, 320 mμ. with $\epsilon_{max.} = 40000$, 7300; in HCl, $\lambda_{max.} = 234$, 325 mμ. with $\epsilon_{max.} = 59000$, 7600.

The determination of the structure of the above glucuronides provides information concerning the orientation of metabolic hydroxylation in the quinoline series. Hydroxylation in position 6 has now been shown to occur with quinoline (Scheunemann, 1923) and 2- and 4-quinolone (cf. I and II). It is to be noted that this position, as far as the benzene ring is concerned, is *para* to the heterocyclic nitrogen atom. This *para*-hydroxylation has also been observed with other heterocyclic compounds such as tryptophan, which is hydroxylated in liver slices to 5-hydroxytryptophan (V) (Udenfriend, Clark & Titus, 1953); phenothiazine, which is hydroxylated in sheep to 3-hydroxyphenothiazine (VI) (leucophenothiazone) (Collier, 1940); and benzoxazolone, which is hydroxylated in rabbits to what is probably 6-hydroxybenzoxazolone (VII) (cf. Bray, Clowes & Thorpe, 1952).

Hydroxylation in the 3-position has now been shown to occur with quinoline (Novack & Brodie, 1950) and with 4-quinolone (cf. III). This suggests that hydroxylation can occur *meta* to the heterocyclic nitrogen atom in the same ring. This also apparently occurs with pyridine and with indole. 3-Hydroxypyridine can be detected chromatographically in the urine of rabbits receiving pyridine (Smith & Williams, unpublished data), and a complex of the glucuronide and the ethereal sulphate of indoxyl has been isolated from the urine of dogs receiving indole (Neuberg & Schwenk, 1917).

In quinoline itself, metabolic hydroxylation also occurs in the 2- and 8-positions (Knox, 1946; Scheunemann, 1923), but the extent to which hydroxylation occurs in the various positions has still to be worked out. In 2-quinolone, the 6-position appears to be the most readily hydroxylated in the rabbit, since paper chromatography of the urine shows only one glucuronide. In 4-quinolone, the 3-position appears to be most readily hydroxylated, but hydroxylation in the 6-position also takes place to a small extent. It is interesting to note that in many of the quinoline antimalarials, for example quinine, the 6-position is usually blocked by a methoxyl group. Cinchonine, which has no 6-methoxyl group, has less than 0·2 of the antimalarial activity of quinine (cf. Sexton, 1953).

It is also interesting to compare the position of metabolic hydroxylation of the quinolones with the orientation of chemical substitution in these compounds. The 2- and 4-quinolones are nitrated with mixed sulphuric and nitric acids mainly in the 6-position; in the absence of sulphuric acid, 4-quinolone is nitrated in the 3-position (Schofield, 1950). Thus, the positions of metabolic hydroxylation of these compounds are similar to those found in nitrations.

SUMMARY

1. The glucuronide obtained by feeding carbostyril (2-quinolone) to rabbits has been proved to be 2-quinolonyl-6-glucosiduronic acid, since it yields 2:6-dihydroxyquinoline on hydrolysis.

2. The crystalline glucuronide (m.p. 208°) obtained in small yield from the urine of rabbits receiving 4-quinolone has been proved to be 4-quinolonyl-6-glucosiduronic acid, since after feeding with 4:6-dihydroxyquinoline the same glucuronide can be isolated.

3. The main glucuronide isolated after feeding with 4-quinolone appears to be 4-quinolonyl-3-glucosiduronic acid, since it yields 3:4-dihydroxyquinoline on hydrolysis.

4. The positions of metabolic hydroxylation of the 2- and 4-quinolones are the same as those found when these compounds are nitrated.

We are grateful to Dr D. Coppini, Modena, Italy, for a sample of 3:4-dihydroxyquinoline. The expenses of this work were in part defrayed by a grant from the Medical Research Council.

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Electrophoretic Studies of Ox Serum

1. THE SERA OF NORMAL CATTLE

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During the course of investigations of the sera of cattle which had been infected with the virus of foot-and-mouth disease or of vesicular stomatitis, a large number of normal sera were analysed electrophoretically. In view of the widespread interest in the proteins of ox serum and the fragmentary nature of published electrophoretic data, it was considered that the present observations from a uniform group of normal cattle should be published in some detail.

MATERIALS AND METHODS

Cattle. The cattle employed were Devon steers from 18 to 30 months old and were 'normal' in the sense that none had suffered from, nor had been exposed to, foot-and-mouth