3. The mechanism of the protective action is explained in terms of the colligative properties of solutions, and desiderata for an ideal protective agent are listed. For human red blood cells the substance most closely fulfilling these criteria is glycerol.

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Biochemical Studies of Toxic Agents

6. THE CONVERSION OF NAPHTHALENE INTO 1:2-DIHYDRO-2-HYDROXY-1-NAPHTHYL GLUCOSIDURONIC ACID IN THE RABBIT

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Rats and rabbits dosed with naphthalene excrete 1:2-dihydronaphthalene-1:2-diol (Young, 1947; Booth & Boyland, 1949), and by analogy with anthracene it might be expected that excretion of a glucuronide of this diol also takes place, for Boyland & Levi (1935, 1936) have shown that in rats and rabbits anthracene is converted into 1:2-dihydroanthracene-1:2-diol, some of which is excreted as a glucuronide (see also Boyland & Wiltshire, 1953). This possibility has been studied in the present work and the results obtained provide good evidence that in the rabbit naphthalene is converted into a glucuronide of 1:2-dihydronaphthalene-1:2-diol, namely 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid.

EXPERIMENTAL

The animals used were male rabbits weighing from 2·5 to 3·0 kg. They were housed in metabolism cages designed to permit the collection of urine separate from the faeces. The animals were fed on a diet consisting of cabbage together with a mixture of bran and chaff and they had access to water at all times.

Quantitative experiments

A quantitative study was made of the excretion of glucuronic acid and ethereal sulphate by rabbits before and after they had been dosed with naphthalene. Two experiments were carried out. In the first the urine of four rabbits was analysed for glucuronic acid and ethereal sulphate on the three consecutive days immediately before the administration of a single dose of naphthalene to each rabbit. Naphthalene (1 g./kg. body wt.) was administered by stomach tube as a 15% (w/v) soln. in liquid paraffin. Daily analysis of the urine was then continued until the glucuronic acid and ethereal sulphate excretion had returned to the pre-dosing level. The second experiment was carried out on another four rabbits in the same way as the first except that 24 hr. after the first dose of naphthalene had been given, each rabbit received a second dose of the same size as the first.

Table 1. *Excretion of inorganic, ethereal, and total sulphate S (IS, ES and TS) and of glucuronic acid in the urine of four rabbits before and after the administration by stomach tube of naphthalene*

(Each dose 1 g./kg. body wt. as a 15% (w/v) soln. in liquid paraffin.)

Day	Sulphate S (mg./kg. body wt.) IS	ES	TS	Glucuronic acid (mg./kg. body wt.)
	Expt. 1. Single dose of naphthalene			
1	24·6	1·7	26·3	13·4
2	34·1	5·2	39·3	22·1
3	22·2	1·7	23·9	31·2
	(Naphthalene administered)			
4	12·1	7·9	20·0	473·0
5	6·0	5·4	11·4	297·0
6	26·2	0·2	26·4	52·6
7	25·3	2·1	27·4	29·0
	Expt. 2. Two doses of naphthalene			
1	30·4	3·0	33·4	26·9
2	24·7	4·2	28·9	20·0
3	21·8	0·8	22·6	29·2
	(Naphthalene administered)			
4	23·3	10·6	33·9	588·0
	(Naphthalene administered)			
5	4·9	6·2	11·1	825·0
6	7·6	4·7	12·3	448·0
7	27·1	3·6	30·7	112·0
8	25·8	2·8	28·6	29·8

Glucuronic acid determinations were carried out by means of the naphthoresorcinol method (Hanson, Mills & Williams, 1944; Bisset, Brooksbank & Haslewood, 1948). A calibration graph was prepared from the results obtained when known amounts of pure 1-naphthyl glucosiduronic acid (previously known as 1-naphthylglucuronide) were heated with naphthoresorcinol in the presence of 3N-HCl in a boiling-water bath for 2 hr. The same conditions were used for the analysis of urine.

Ethereal sulphate excretion was obtained from the difference between the inorganic sulphate and total sulphate excretions and these values were determined by means of the benzidine-precipitation procedure (cf. Laidlaw & Young, 1953). The benzidine sulphate precipitates were filtered off and washed, using an apparatus of the type described by Henriques, Kistiakowsky, Margnetti & Schneider (1946), and they were then titrated with 0·02N-NaOH, using phenol red as indicator.

The results are shown in Table 1. The increase in ethereal sulphate excretion which occurred after the administration of naphthalene was small and of doubtful significance. On the other hand, the excretion of glucuronic acid was markedly increased, and on the basis of one molecule of glucuronic acid per molecule of naphthalene the increase corresponded to about 50% of the naphthalene administered when a single dose was given and to about 60% of the dose when two doses were given.

Separation of a glucuronide from the urine of rabbits dosed with naphthalene

A series of experiments was carried out in each of which eight rabbits were each given a dose of naphthalene by stomach tube, followed by a second dose 24 hr. later. Each rabbit received in each dose 1 g. naphthalene/kg. body wt. in the form of a 15% (w/v) soln. in liquid paraffin. The urine (pH 7–8) was collected for 72 hr. after giving the first dose, filtered through glass wool and evaporated to 500–600 ml. under reduced pressure. After the concentrate had been adjusted to pH 6–7 animal charcoal (30 g.), which had just been heated strongly and allowed to cool to room temperature, was added with vigorous stirring. The mixture was immediately filtered on a Büchner funnel and a further 30 g. of charcoal were added to the filtrate. The addition of charcoal followed by filtration was continued until 180 g. of charcoal had been used. Analysis showed that this procedure removed 80–90% of the glucuronide present in the urine.

When the charcoal was dry, it was split into six 30 g. portions, each of which was stirred vigorously with 300 ml. of hot methanol. The mixtures were filtered, and the methanol filtrates were combined and evaporated under reduced pressure. This yielded a red gum which was treated with a small volume of water and filtered to remove a small amount of insoluble material. The filtrate was concentrated under reduced pressure to a viscous, red gum. This was stirred into a large excess of ethanol and immediately a copious, pale-brown precipitate separated. The precipitate was filtered off and dried over anhydrous $CaCl_2$ *in vacuo* giving a brownish white powder (15–20 g.). When the ethanol filtrate was evaporated to dryness under reduced pressure, redissolved in a minimum of water and stirred into an excess of ethanol, a further small quantity of pale-brown precipitate separated and was added to that already obtained. This powder was rich in glucuronide and is referred to hereafter as the 'glucuronide fraction.'

Properties of the glucuronide fraction. The powder was hygroscopic and on exposure to air it was quickly converted into a red gum. It dissolved readily in water to give a brown, neutral solution. It had a low solubility in warm methanol. A faint, pink colour was produced when a solution of diazotized sulphanilic acid was added to 3 ml. of a 0·1% aqueous solution of the powder containing an excess of NaOH. A deep-red colour formed when excess of NaOH followed by diazotized sulphanilic acid was added to 3 ml. of a 0·1% solution of the powder in N-HCl after it had been heated in a boiling-water bath for 15 min. When a 0·1% solution of the powder in 5N-HCl was heated with naphthoresorcinol in a boiling-water bath for 15 min. it gave a copious, dark-blue precipitate soluble in amyl alcohol. These findings indicated the presence of a trace of free naphthol in the powder, together with a compound or compounds which yielded naphthol and glucuronic acid on treatment with acid.

Action of β-glucuronidase on the glucuronide fraction. In order to gain information concerning the nature of the glucuronide(s) present the glucuronide fraction was subjected to the action of β-glucuronidase. The enzyme was prepared from mouse liver by a method based on that of Kerr & Levvy (1951) as follows. Fresh liver (6 g.) from four male white mice was comminuted in 30 ml. of water for 1 min. in an ice bath. To the dispersion were added 30 ml. of 0·2M acetate buffer at pH 5·2. The mixture was centrifuged at 1500 *g* for 15 min., and the sedimented material was washed with 10 ml. of 0·1M buffer and again centrifuged. The supernatant solution together with the washing served as the enzyme preparation.

The glucuronide powder was washed thoroughly with ether and, after it had been freed from ether, 4 g. of it were dissolved in 80 ml. of 0·1M acetate buffer at pH 5·2. The solution was divided into two equal parts. To one half was added the glucuronidase preparation. The other half was made up to 100 ml. by the addition of 60 ml. of buffer at pH 5·2. Both solutions were incubated at 37–38° for 24 hr. Then 2 ml. of 2N-NaOH were added to each solution, a small amount of precipitate was removed by filtration, and the filtrate was extracted with peroxide-free ether in a continuous extractor for 24 hr.

When the ether extract of the solution which contained glucuronidase was evaporated to dryness it yielded a pink, crystalline residue. When a small amount of this residue was treated with diazotized sulphanilic acid in the presence of an excess of NaOH, a pale-pink colour was produced. When this test was applied to a small amount of residue which had been heated in 2N-HCl, a deep, cherry-red colour was obtained. These observations suggest that the ether extract contained a trace of naphthol and a considerable amount of a dihydronaphthalenediol or some substance which yields naphthol when treated with HCl. Tests for the presence of glucuronide and ethereal sulphate gave negative results.

When similar tests were applied to the residue obtained from evaporation of the ether extract of the solution which did not contain glucuronidase, a faintly positive diazo test for naphthol was obtained, but this was not intensified when the residue was first heated in acid solution, and it was apparent, therefore, that this residue did not contain a dihydronaphthalenediol.

The residue obtained by evaporation of the ether extract of the enzyme-treated preparation was dried thoroughly and was then refluxed with 50 ml. *cyclo*hexane for 30 min. The *cyclo*hexane extract was separated and on cooling

yielded 0·130 g. of pink crystals, m.p. 124–125° (this and other m.p.'s reported herein are uncorrected) which were crystallized from benzene and yielded colourless needles, m.p. 127–128°, $[\alpha]_D^{20}$ +157° in ethanol (*c*, 1). (Found: C, 73·5; H, 6·0. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%). The compound (0·060 g.) was refluxed for 15 min. with 2 ml. of pyridine and 1 ml. of acetic anhydride. The solution was poured into 10 ml. of ice-water and the precipitate which formed was crystallized from aqueous methanol. The product obtained (0·053 g.) melted at 77°, $[\alpha]_D^{22}$ +421° in ethanol (*c*, 0·5). (Found: C, 68·8; H, 6·1. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%). Booth & Boyland (1949) isolated (+)-1:2-dihydronaphthalene-1:2-diol from the urine of rabbits, m.p. 125°, $[\alpha]_D^{22}$ +159° in ethanol (*c*, 1). They also prepared the diacetate of this compound, m.p. 77°, $[\alpha]_D^{22}$ +437° in ethanol (*c*, 0·5–1·0).

From the experiments just described it was apparent that whereas the glucuronide fraction contained a trace of naphthol but no free dihydronaphthalenediol, incubation at pH 5·2 with glucuronidase brought about no significant increase in the amount of naphthol present but liberated much (+)-1:2-dihydronaphthalene-1:2-diol. It appeared probable, therefore, that the powder contained a glucuronide of this diol.

Acid decomposition of the glucuronide fraction. It is known that 1- and 2-naphthyl glucosiduronic acids can be precipitated from aqueous solution in the form of their *p*-toluidine salts (Berenbom & Young, 1951), and this was used in examining the glucuronide fraction for the presence of naphthyl glucosiduronic acid before and after it had been subjected to acid decomposition.

A solution of 2 g. of the glucuronide powder in 10 ml. of water, pH 6–7, was warmed to about 70° and *p*-toluidine hydrochloride (0·3 g.) was added. The mixture was stirred vigorously and a clear solution was obtained. No precipitate formed on cooling.

A further 2 g. of the glucuronide powder was dissolved in 10 ml. of water and the solution was brought to pH 1 by addition of conc. HCl. The solution was heated in a boiling-water bath for 5 min. and after it had been cooled it was adjusted to pH 6–7 by addition of 2N-NaOH. The solution was warmed to about 70° and *p*-toluidine hydrochloride (0·3 g.) was added with stirring. Almost immediately a copious, crystalline precipitate formed. The solution was cooled to room temperature and the precipitate was filtered off, dissolved in 20 ml. of hot water, and charcoal was added. The charcoal was removed by filtration, and the filtrate, on cooling, yielded a bulky, white, crystalline precipitate. This was separated and after it had been dried over P_2O_5 *in vacuo* it weighed 0·320 g. The compound was recrystallized from water and 0·150 g. of the product was dissolved in 1·5 ml. 6N-HCl, and a precipitate formed when the solution was stirred. The mixture was cooled in the refrigerator and the precipitate was separated by filtration. It was recrystallized from water and yielded 0·045 g. of colourless crystals, m.p. 198–200°, and gave positive tests for naphthol and glucuronic acid when hydrolysed by acid. When the compound was mixed with an authentic specimen of 1-naphthyl glucosiduronic acid (m.p. 199°), the m.p. was not depressed, $[\alpha]_D^{20}$ −84° in ethanol (*c*, 1). For 1-naphthyl glucosiduronic acid, Berenbom & Young (1951) reported $[\alpha]_D^{20}$ −85° in ethanol (*c*, 1).

It can be concluded from these experiments that if the glucuronide fraction contained any 1-naphthyl glucosiduronic acid the amount was small. This compound was formed, however, by acid decomposition of a glucuronide present in the powder. By analogy with the behaviour of 1:2-dihydro-2-hydroxy-1-anthryl glucosiduronic acid (cf. Boyland & Levi, 1936) it is to be expected that the acid decomposition of the corresponding derivative of 1:2-dihydronaphthalene-1:2-diol would lead to the formation of 1-naphthyl glucosiduronic acid.

Preparation of methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri-O-acetylglucosid)uronate. A solution of 2 g. of the glucuronide powder in 40 ml. of warm methanol was cooled to 4°, filtered, and an ethereal solution of diazomethane freshly prepared from 7 g. of nitrosomethylurea was added. The mixture was allowed to stand overnight in the refrigerator and a small precipitate which formed was removed by filtration. The filtrate was evaporated under reduced pressure and the pale-brown solid which remained was dissolved in methanol and again allowed to react with diazomethane freshly prepared from 7 g. of nitrosomethylurea. After the mixture had stood for 12 hr. in the refrigerator it was evaporated to dryness under reduced pressure and yielded a pale-brown, neutral material soluble in ether and slightly soluble in water. Attempts to induce this substance to crystallize were unsuccessful. It was dissolved in 7 ml. of pyridine and 5 ml. of acetic anhydride were added. The mixture was allowed to stand at room temperature for 3 days and it was then stirred into 100 ml. of ice-water. Almost immediately a precipitate formed. After the mixture had been allowed to stand in the refrigerator for 2 hr., the pale-yellow precipitate was filtered off and after crystallization from aqueous ethanol 0·206 g. of product was obtained. This was crystallized once from acetone and twice from aqueous ethanol, and the product was dried to constant weight over P_2O_5 *in vacuo*. The compound melted at 209° and after further crystallization from aqueous ethanol the m.p. was unchanged. The long, colourless needles were readily soluble in $CHCl_3$, warm acetone, and warm ethanol, but insoluble in water. $[\alpha]_D^{20}$ +94° in $CHCl_3$ (*c*, 1). The analysis of the compound corresponded to that of *methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri-O-acetylglucosid)uronate.* (Found: C, 57·5, 57·8; H, 5·6, 5·6; CH_3CO—, 31·5. $C_{25}H_{28}O_{12}$ requires C, 57·7; H, 5·5; CH_3CO—, 33·0%.) The compound gave strongly positive tests for naphthol and glucuronic acid after it had been heated in HCl soln. in a boiling-water bath.

Preparation of derivatives of 1- *and* 2-*naphthyl glucosiduronic acid*

As the isolation of 1-naphthyl glucosiduronic acid from the urine of dogs and rabbits dosed with naphthalene has been reported (Lesnik, 1888; Masamune, 1937) it was decided to prepare, for purposes of comparison, acetylated methyl esters of 1- and 2-naphthyl glucosiduronic acid. Rabbits were dosed with 1- and 2-naphthol, and 1- and 2-naphthyl glucosiduronic acids were isolated from the urine by the procedure described by Berenbom & Young (1951).

Methyl (1-naphthyl tri-O-acetylglucosid)uronate. Four male rabbits (average body wt. 2·6 kg.) were each given 0·5 g. 1-naphthol/kg. body wt. on 2 successive days. The naphthol was given by stomach tube as a suspension in 1% starch solution. The urine was collected for 72 hr. after the first dosing and from it was separated 4·514 g. of *p*-toluidine (1-naphthyl glucosid)uronate. This accounted for 14·7% of the naphthol administered. The *p*-toluidine salt was decomposed and yielded 2·251 g. of 1-naphthyl glucosiduronic

acid, $[\alpha]_D^{19} - 85°$ in ethanol (*c*, 1), m.p. 199°. The mixed m.p. with a specimen of 1-naphthyl glucosiduronic acid isolated from rat urine (Berenbom & Young, 1951) was identical.

To 0·250 g. of 1-naphthyl glucosiduronic acid dissolved in 5 ml. of ethanol was added an ethereal solution of diazomethane until the mixture showed a persistent, yellow colour. The mixture was allowed to stand overnight. The solvents were then removed by evaporation and the residue was dried over P_2O_5 *in vacuo*. This yielded 0·139 g. of a white powder, m.p. 165°, which was neutral in reaction, soluble in ethanol and almost insoluble in water. It was dissolved in 2 ml. of pyridine and 1 ml. of acetic anhydride was added. After the mixture had been allowed to stand at room temperature for 3 days it was poured, with stirring, into 20 ml. of ice-water. A colourless, crystalline product was obtained which was recrystallized from ethanol, and yielded 0·125 g. of *methyl (1-naphthyl tri-O-acetylglucosid)uronate*, m.p. 160°, $[\alpha]_D^{20} - 79°$ in $CHCl_3$ (*c*, 1). (Found: C, 59·5; H, 5·7; CH_3CO—, 27·7. $C_{23}H_{24}O_{10}$ requires C, 60·0; H, 5·2; CH_3CO—, 28·0%.) The compound was soluble in $CHCl_3$, warm acetone, and warm ethanol. It was insoluble in water. It gave positive tests for naphthol and for glucuronic acid after it had been heated with acid.

Methyl (2-naphthyl tri-O-acetylglucosid)uronate. The procedure used for obtaining 2-naphthyl glucosiduronic acid closely resembled that just described for 1-naphthyl glucosiduronic acid. Four male rabbits received a total of 10·1 g. of 2-naphthol by stomach tube and 3·107 g. of *p*-toluidine (2-naphthyl glucosid)uronate were separated from the urine. This corresponded to 10·5% of the 2-naphthol administered. Treatment of the *p*-toluidine salt with HCl yielded 2-naphthyl glucosiduronic acid. After drying over P_2O_5 *in vacuo* this weighed 2·472 g., m.p. 153–154°, $[\alpha]_D^{20} - 96°$ in ethanol (*c*, 1).

2-Naphthyl glucosiduronic acid (0·200 g.) was converted into its methyl ester (0·160 g.) and this was acetylated as was 1-naphthyl glucosiduronic acid. The yield of *methyl (2-naphthyl tri-O-acetylglucosid)uronate* was 0·140 g., m.p. 127–128°, $[\alpha]_D^{20} - 35°$ in $CHCl_3$ (*c*, 1). (Found: C, 60·4; H, 5·3; CH_3CO—, 27·6. $C_{23}H_{24}O_{10}$ requires C, 60·0; H, 5·2; CH_3CO—, 28·0%.) The compound gave positive tests for naphthol and glucuronic acid after it had been heated with acid.

DISCUSSION

From the urine of a dog dosed with naphthalene, Lesnik (1888) isolated 1-naphthyl glucosiduronic acid. In one experiment of a series in which he administered naphthalene to rabbits, Masamune (1937) obtained a laevorotatory compound which proved to be 1-naphthyl glucosiduronic acid, but in all other experiments he obtained a dextrorotatory compound which was described by Umezawa & Masamune (1938) as 'a kind of α-naphtholglucuronic acid' and appeared to be a hydrate of 1-naphthyl glucosiduronic acid, $C_{16}H_{16}O_7$, $1·5\,H_2O$ (Umezawa & Masamune, 1938). As the 1-naphthyl glucosiduronic acid isolated from the urine of animals dosed with 1-naphthol is a laevorotatory compound, it has been suggested (Young, 1950) that it is not unlikely that the dextrorotatory compound isolated by Masamune from rabbit urine was the glucuronide of 1:2-dihydronaphthalene-1:2-diol.

In the present work it was found that glucuronide excretion is greatly increased after the administration of naphthalene to rabbits, and from the urine of the dosed animals a fraction rich in glucuronide was obtained. Attempts to isolate a pure glucuronide from this fraction resulted in the formation of gums. By treating the fraction with glucuronidase it was possible to demonstrate the liberation of dextrorotatory 1:2-dihydronaphthalene-1:2-diol. Decomposition of the fraction with acid led to the formation of 1-naphthyl glucosiduronic acid. These findings are in accordance with the presence in the fraction of 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid. This was confirmed by the preparation from the glucuronide fraction of a tetraacetate methyl ester—presumably methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri-*O*-acetylglucosid)uronate. This compound had a melting point and optical rotation markedly different from those of methyl (1- and 2-naphthyl

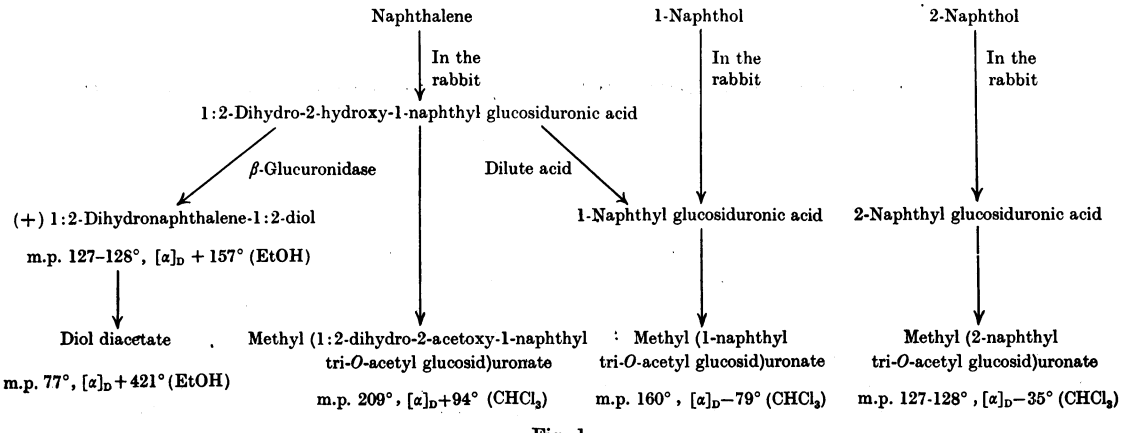

Fig. 1.

tri-*O*-acetylglucosid)uronates. The various reactions studied are summarized in Fig. 1.

In addition to 1-naphthyl glucosiduronic acid there is now evidence for the presence in the urine of naphthalene-dosed animals of two other glucuronic acid derivatives of naphthalene metabolites. As well as undergoing conversion into 1:2-dihydronaphthalene-1:2-diol and the glucuronide of this compound, naphthalene is converted into a derivative which yields naphthalene on acidification. It has been suggested that this might be a compound of the dihydronaphthol type (Bourne & Young, 1934). Recently Boyland & Solomon (1953) have prepared from the urine of rats and rabbits dosed with naphthalene a compound corresponding to 'a triacetyl derivative of 1:2-dihydronaphthol glucuronide methyl ester', m.p. 203°, $[\alpha]_D^{20} + 23°$ in chloroform (*c*, 1).

Although no evidence has been obtained in the present work for the occurrence of 1-naphthyl glucosiduronic acid in the urine of rabbits dosed with naphthalene, this compound may nevertheless be present. The ease with which 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid loses the elements of water under acidic conditions, however, may account for some, at least, of the 1-naphthyl glucosiduronic acid reported by previous workers to be present in the urine of animals dosed with naphthalene.

SUMMARY

1. The glucuronide content of the urine of rabbits is greatly increased after the administration of naphthalene by stomach tube.

2. A glucuronide fraction has been separated from the urine of rabbits dosed with naphthalene, and treatment of this fraction with hot, dilute acid gave 1-naphthyl glucosiduronic acid. Incubation of the glucuronide fraction with β-glucuronidase liberated dextrorotatory 1:2-dihydronaphthalene-1:2-diol.

3. These findings are consistent with the presence of 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid in the glucuronide fraction, and this has been confirmed by the preparation of a tetraacetate methyl ester, methyl (1:2-dihydro-2-acetoxy-1-naphthyl tri-*O*-acetylglucosid)uronate, from this fraction.

4. From the urine of rabbits dosed with 1- and 2-naphthol by stomach tube, it has been possible to isolate 1- and 2-naphthyl glucosiduronic acid, respectively. Methyl (1- and 2-naphthyl tri-*O*-acetylglucosid)uronates have been prepared.

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Activation of the Succinic Dehydrogenase-cytochrome System

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The object of this paper is the study of the activation by various agents of the succinic dehydrogenase-cytochrome system of different tissue preparations. Such preparations will be briefly referred to as succinic oxidase preparations. Keilin & Hartree (1947, 1949) have shown that succinic oxidase preparations which had been obtained from a variety of sources and which had been inactivated by different means could be reactivated by such relatively inert substances as denatured globin and tricalcium phosphate gel. They found that several proteins exhibited the same property, but denatured globin was by far the most effective. It was also shown that succinic oxidase preparations

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