Mycobacterium smegmatis at a concentration of 1:20,000 and *Myco. phlei* at 1:10,000 but was completely inactive against an unidentified species of *Mycobacterium* not listed in Table 1.

Fuscin-thioglycollic acid. The results obtained are summarized in Table 2. They indicate that, in general, the conversion of fuscin into its thioglycollic acid derivative caused a remarkably small change in its antibacterial spectrum, cf. Table 1.

SUMMARY

1. Fuscin is a somewhat powerful antibacterial substance, being particularly active against Gram-positive bacteria; but much less active or substantially inactive against mycobacteria and Gram-negative bacteria.

2. The conversion of fuscin into its thioglycollic acid derivative causes a remarkably small change in its antibacterial spectrum.

Table 2. Antibacterial activity of fuscin-thioglycollic acid

Micro-organism	Catalogue no.	Limiting dilution for inhibition of growth	
		Complete	Partial
<i>Escherichia coli</i>	N.C.T.C. 86	—	5,000
<i>Proteus vulgaris</i>	N.C.T.C. 5887	5,000	—
<i>Vibrio cholerae</i>	N.C.T.C. 1633	20,000	—
<i>Bacillus subtilis</i>	L.S.H.	40,000	80,000
<i>Staphylococcus aureus</i>	N.C.T.C. 3093	40,000	160,000
<i>Staph. aureus</i>	L.S.H. 2	40,000	80,000
<i>Staph. aureus</i>	Oxford H	160,000	320,000
<i>Staph. aureus</i>	L.S.H. 1	160,000	320,000
<i>Bacillus anthracis</i>	N.C.T.C. 5444	640,000	1,280,000
<i>Corynebacterium diphtheriae mitis</i>	L.S.H.	640,000	1,280,000

Metabolism of Fluorene in the Rabbit

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(Received 3 May 1948)

The metabolism of the hydrocarbon fluorene does not appear to have been examined previously, although the compound has been investigated for carcinogenic activity in skin-painting experiments with mice (Bloch, 1922, quoted by Kennaway, 1930; Twort & Fulton, 1930) and more recently in feeding experiments with rats (Wilson, De Eds & Cox, 1947). The results of these experiments led to the conclusion that fluorene is not carcinogenic. The high carcinogenic potency of 2-acetamidofluorene is now well established, as may be seen from the review by Bielschowsky (1947), but the only publication which has appeared to date concerning the metabolism of this carcinogen is that of Bielschowsky (1945) who showed that the metabolite, 7-hydroxy-2-acetamidofluorene, may be isolated from the urine of rats fed 2-acetamidofluorene.

In the course of further studies of the metabolism of 2-acetamidofluorene in rabbits an investigation was made concerning the fate of fluorene in these animals with the primary object of determining whether the reactive 9-methylene group of this compound could undergo any change during the passage

of the hydrocarbon through the animal body. This work might lead to a better insight into the metabolism of the related amine carcinogen. It was also of interest to see if fluorene is metabolized by rabbits according to the hydroxylation mechanism which has been found to play a part in the detoxication of a number of carcinogenic and non-carcinogenic polycyclic hydrocarbons. Adequate reviews of this detoxication mechanism have been given by Boyland & Weigert (1947) and by Williams (1947); only one example will be quoted here. Young (1947) found that the urine from rats ingesting naphthalene contained the diol, 1:2-dihydroxy-1:2-dihydronaphthalene, which yielded α -naphthol on mild acid hydrolysis, and Bourne & Young (1933) showed that naphthalene in man and dog gave rise to α -naphthol and the glucuronide of this compound. These results are in accord with the views of Fieser (1941) that the detoxication reaction involves primarily the addition of the elements of hydrogen peroxide to the hydrocarbon molecule in a perhydroxylation reaction giving rise to diols which are usually unstable and which pass over into phenolic compounds by

loss of water from the diol grouping. With regard to the positions in the hydrocarbon nucleus at which *in vivo* hydroxylation occurs the interesting observation was made by Berenblum & Schoental (1943) that benzantracene and dibenzanthracene are metabolically attacked (hydroxylated) at points where sulphonation of these hydrocarbons occurs *in vitro*, and Bielschowsky showed that this rule applied to 2-acetamidofluorene which is metabolized by rats to the 7-hydroxy compound, 2-aminofluorene being sulphonated *in vitro* at position 7 according to Courtot (1930).

Although no evidence has been found that the reactive methylene grouping of fluorene is attacked *in vivo* it has been shown that 2-hydroxyfluorene and a compound which analyzes correctly as the glucuronide of this phenol can be isolated from the urine of rabbits ingesting fluorene.

EXPERIMENTAL

Materials and methods. The fluorene sample employed in this work was obtained by crystallizing a commercial fluorene preparation from ethanol. It melted at 113°. For an experiment in which 2-hydroxyfluorene was fed to a rabbit, this phenol (m.p. = 168°) was prepared from 2-aminofluorene according to the method of Diels (1901).

The detection and identification of fluorene derivatives occurring in the urine of rabbits ingesting fluorene compounds was facilitated by means of sensitive colour reactions given by traces of fluorene derivatives when these are treated with drops of a solution of *p*-dimethylaminobenzaldehyde (5% w/v) in conc. H₂SO₄. These colour reactions are described in the text and the use of the reaction will be discussed more fully in another communication. All melting points are uncorrected. Carbon and hydrogen analyses were carried out by Drs Weiler and Strauss (Oxford).

RESULTS

Tolerance of the rabbit to fluorene. A female rabbit which had not been used for any other experiment was maintained on a stock diet consisting of oats (1 part), sharps (1 part), bran (2 parts) and milk powder (2 parts). The animal received 70 g./day of this diet in which was incorporated 250 mg. of fluorene, the diet being mixed thoroughly with the addition of water. 100 g. of cabbage was fed daily. The animal received 1.5 g. of fluorene (at 250 mg./day) and 10 days after this experiment it received 1 g. of fluorene at the same dosage level/day. The animal gained in weight over the period of the experiment.

In another experiment in which a male rabbit (initial weight, 2120 g.) was fed fluorene at the level of 250 mg./day in 70 g. of stock diet for 20 consecutive days an increase in body weight of 400 g. was observed over this period. The animal appeared to be in good health and 2 months after the end of the

experiment it weighed 2450 g. It would appear then that rabbits tolerate fluorene well and that ingestion of the compound for short periods at least has no serious effect on the health of the animals.

The animals receiving fluorene were kept in metabolism cages, the urine being collected daily and preserved with chloroform in a refrigerator. The urine contained appreciable amounts of phosphate and gave a positive Tollens test for glucuronic acid. When several drops of the urine were placed in a depression in a glazed white tile and treated with 0.25 ml. of *p*-dimethylaminobenzaldehyde reagent a bright purple-pink coloration developed which soon changed to green on standing. This colour reaction indicates the presence of fluorene derivatives, normal rabbit urine giving no purple colour with the reagent.

Isolation of 2-hydroxyfluorene. In an experiment in which 1.75 g. of fluorene was fed to a rabbit at the rate of 250 mg./day, 900 ml. of urine were collected. The urine, which was alkaline, was divided into three portions of 300 ml., each of which was extracted five times with 100 ml. of ether. To obtain a satisfactory separation, the ether-urine emulsions were centrifuged and the combined ether extracts were dried over anhydrous Na₂SO₄. The ether extract was concentrated to 10 ml., poured into a small flask containing about 15 ml. of water and the flask warmed carefully until all the ether had been expelled. Solid material separated giving a purple coloration when treated with a drop of 5% (w/v) *p*-dimethylaminobenzaldehyde in concentrated sulphuric acid. The same colour reaction was given by synthetic 2-hydroxyfluorene. The solid product was filtered and dried at the pump. It was then shaken thoroughly with cold dilute caustic soda solution to dissolve as much of the product as possible, and filtered. The dark coloured filtrate was acidified with concentrated hydrochloric acid and the resulting crude brown precipitate was washed with water and dried at 37°. This product (100 mg.) was crystallized several times from water in the presence of activated charcoal, and a white substance (40 mg.) was obtained which, after drying, had m.p. 167–168°, and mixed m.p. 168° with a sample of synthetic 2-hydroxyfluorene. (Found: C, 85.53; H, 5.64. Calc. for C₁₃H₁₀O: C, 85.67; H, 5.55%.)

About 30 mg. of biosynthetic 2-hydroxyfluorene was dissolved in dilute NaOH and the solution treated with a drop of dimethyl sulphate. The white precipitate which appeared after a time was collected and crystallized from ethanol-water. It melted at 100–104°, mixed m.p. 104° with 2-methoxyfluorene m.p. 104° (Werner, 1902, gives 106–108°), prepared by treating synthetic 2-hydroxyfluorene with dimethyl sulphate. Both specimens of methoxy compound gave the same ink-blue coloration when traces

were treated with a drop of *p*-dimethylaminobenzaldehyde reagent. The colour developed is quite distinct from the purple colour given by 2-hydroxyfluorene in this test. Pure samples of 2-hydroxyfluorene and 2-methoxyfluorene gave no colour reaction with concentrated H_2SO_4 only.

Isolation of a glucuronide. The urine which had been freed from 2-hydroxyfluorene by ether extraction was acidified with concentrated hydrochloric acid, about 20 ml. of acid being added to 300 ml. of urine (slightly more acid than is required to make the urine acid to Congo red). Ether extraction was performed as described for the alkaline urine, but the extraction was incomplete since ether-soluble orange-coloured material could still be obtained by further extraction of the urine with ether. The ethereal extract was orange-red in colour and after removal of the ether a dark red oil was obtained. A small amount of this oil gave a good Tollens reaction for glucuronic acid. The red oil was suspended in about 10 ml. of water and the suspension boiled in the presence of activated charcoal. On cooling the filtrate, white needles separated. After two more crystallizations of this material from water, the colourless filtrate yielded fine long white needles on cooling. This product, which gave a positive reaction for glucuronic acid, was dried at 37° overnight. It melted at 214–215°. The compound analyzed as the *dihydrate* of *fluorene-2-glucuronide*:

(Found: C, 58.1; H, 5.57. $\text{C}_{18}\text{H}_{18}\text{O}_7 \cdot 2\text{H}_2\text{O}$ requires C, 57.84; H, 5.63 %.) After drying *in vacuo* at 100°, anhydrous *fluorene-2-glucuronide* was obtained. (Found: C, 62.36; H, 5.46. $\text{C}_{18}\text{H}_{16}\text{O}_7$ requires C, 63.66; H, 5.07 %.) When traces of the material were treated with a drop of concentrated sulphuric acid the crystals became dark green, and after mixing with a glass rod black particles were observed in a pale green solution. With a drop of *p*-dimethylaminobenzaldehyde reagent the crystals became bright purple-blue (reminiscent of the purple solution given by 2-hydroxyfluorene with the aldehyde reagent) finally yielding black particles in pale purple sulphuric acid medium.

Continuous extraction of acid urine. Very satisfactory yields of the glucuronide were obtained when 300 ml. of acidified urine (previously extracted when alkaline to remove 2-hydroxyfluorene) were subjected to continuous extraction with ether in a Kutscher-Steudel apparatus. After several hours, long white needles separated on the walls of the extraction flask just above the level of the boiling ether, and within 15–20 hr., quite a large amount of fairly pure glucuronide was obtained. The product was removed from the flask and after one crystal-

lization from water and drying overnight at 37° was obtained as fine long white needles melting at 214–215°. The yield of pure compound was 100 mg. It was sparingly soluble in ether, insoluble in benzene, slightly soluble in ethanol, and quite soluble in dilute sodium carbonate. On drying *in vacuo* at 100° to constant weight, 3.908 mg. glucuronide lost 0.255 mg. (6.5 % H_2O). Theoretical loss for $1\text{H}_2\text{O}$, 4.8 %; for $2\text{H}_2\text{O}$, 9.1 %. The compound analyzed as a *hydrate* of *fluorene-2-glucuronide*. (Found: C, 59.92, H, 5.92. $\text{C}_{18}\text{H}_{18}\text{O}_7 \cdot 1\frac{1}{2}\text{H}_2\text{O}$ requires C, 59.1; H, 5.45 %.) After drying to constant weight *in vacuo* at 100°, *fluorene-2-glucuronide* was obtained. (Found: C, 63.62; H, 5.24. $\text{C}_{18}\text{H}_{16}\text{O}_7$ requires C, 63.66; H, 5.07 %.) The compound appears to have a low specific rotation. 5 ml. of a solution of the glucuronide dihydrate (5 mg./ml.), prepared by dissolving the glucuronide in a solution of sodium carbonate containing sufficient alkali to form the monosodium salt, gave in a 1 dm. tube a very small laevorotation (-0.05°). There can be little doubt that the compound is the dihydrate of the glucuronide of an hydroxyfluorene which is thought to be the 2-hydroxy compound. Unsuccessful attempts have been made to isolate this phenol from the products of acid hydrolysis of the glucuronide. It is thought that hydrolytic products of the glucuronide are condensing with hydroxyfluorene at the 9-methylene group thus preventing isolation of the hydroxyfluorene.

A note on the metabolism of 2-hydroxyfluorene in the rabbit

In the hope of obtaining a glucuronide of 2-hydroxyfluorene for comparison with the glucuronide isolated from the urine of rabbits ingesting fluorene, it was decided to examine the metabolism of 2-hydroxyfluorene fed at the level of 250 mg./day in 70 g. stock diet to a male rabbit. The animal tolerated the material well and in the course of 10 days, during which time it ingested 2.5 g. of 2-hydroxyfluorene, it steadily gained in weight from 1670 to 1840 g. Five days after the experiment was terminated the rabbit weighed 1980 g. The urine from this animal gave a positive Tollens reaction and when 0.25 ml. of urine was treated with 0.25 ml. of *p*-dimethylaminobenzaldehyde reagent the bright purple colour characteristic of 2-hydroxyfluorene was obtained. With conc. H_2SO_4 only the urine gave no coloration. 2-Hydroxyfluorene could be isolated readily from the alkaline urine by ether extraction, but no solid glucuronide separated from the acidified urine (20 ml. conc. hydrochloric acid/300 ml. urine) submitted to continuous extraction with ether. The ether extract, however, gave a positive reaction for glucuronic acid. It would appear that 2-hydroxyfluorene is excreted for the most part unchanged. Certainly

there is very little conjugation with glucuronic acid. The urine was not examined for the presence of ethereal sulphates.

A note on the occurrence of fluorene in faeces

About 40 g. of faeces were collected from a rabbit which was receiving 250 mg. fluorene/day. The faeces were allowed to dry at room temperature for several days and then ground to a powder with an equal weight of anhydrous sodium sulphate. The powder was ground under ether repeatedly and after each extraction the ether was filtered off, the combined ethereal extracts concentrated, dried over anhydrous sodium sulphate and evaporated to dryness. The dark green residue was taken up in light petroleum (b.p. 40–60°) and chromatographed on activated alumina (British Drug Houses product). All pigments remained on the column and the petroleum ether eluate was colourless. A drop of this eluate was allowed to evaporate on a glazed white tile and the residue treated with a drop of *p*-dimethylamino-benzaldehyde reagent. A bright-pink colour very like that given by fluorene developed. No colour appeared when a similar experiment was carried out using instead a drop of concentrated sulphuric acid. Several other experiments were carried out with faeces collected at different times and whilst the light petroleum extract sometimes gave a positive reaction for fluorene, on other occasions it did not. It is concluded that little if any fluorene is excreted in the faeces, the positive reaction for fluorene sometimes observed being due probably to contamination of the faeces with the hydrocarbon from the food. The reaction for fluorene is extremely sensitive; as little as 1 µg. may be detected readily.

DISCUSSION

The metabolites obtained from the urine of rabbits ingesting fluorene are 2-hydroxyfluorene and the glucuronide of this phenol. Data relating to the yields of these metabolites were accidentally destroyed so that only a rough estimate of the recoveries can be made. In the urine collected from a rabbit which had ingested 1.75 g. of fluorene about 40 mg. of pure 2-hydroxyfluorene was obtained, and by continuous extraction with ether the acidified urine yielded approximately 400–500 mg. of pure glucuronide (computed from recoveries obtained from fractions of the total urine). Both metabolites were isolated in the above proportions from the separate urines of two rabbits ingesting fluorene. Little if any un-

changed fluorene is excreted in the faeces and this appears to be in agreement with the work of Chang (1943), who showed that only small amounts of the simple hydrocarbons such as naphthalene and phenanthrene are eliminated in the faeces of rats receiving these compounds, whereas considerable amounts of the more complex hydrocarbons such as methylcholanthrene are excreted by this route.

The metabolism of the hydrocarbon follows the rule stated by Berenblum & Schoental (1943) (and confirmed by Bielschowsky (1945) for the case of 2-acetamidofluorene) that the site of biological hydroxylation is that at which sulphonation occurs *in vitro*. Courtot & Geoffroy (1924) showed that fluorene-2-sulphonic acid is the first product of sulphonation of the hydrocarbon *in vitro*.

It is possible that 2-hydroxyfluorene arose from diols during the isolation of the compound from the urine, although the crude solid extract after decolorization by charcoal was found to be optically inactive, whereas diols of fluorene, if present, would be expected to show optical activity.

With regard to the tolerance of the rabbit to fluorene it is interesting to note that Wilson *et al.* (1947) found that groups of rats maintained on a diet containing fluorene at the levels of 0.062–0.5 % for 106 days were in good health after 453 days and at autopsy they showed no signs of gross tissue damage. The growth rate of rats receiving fluorene at concentrations of 0.5–1 % was impaired, but the health of these animals appeared to be good.

SUMMARY

1. Two metabolites of fluorene have been isolated from the urine of rabbits, viz. 2-hydroxyfluorene and a compound which analyzes correctly for the glucuronide of this phenol. This is a further example of the biological hydroxylation of a hydrocarbon taking place at the point at which sulphonation occurs *in vitro*.
2. Little if any fluorene is eliminated in the faeces and no metabolites of the hydrocarbon have been detected in these.
3. 2-Hydroxyfluorene fed to a rabbit appears to be excreted largely unchanged.
4. Fluorene and 2-hydroxyfluorene are well tolerated by the rabbit.

I wish to express my best thanks to Prof. H. N. Green for his interest in this work and to Mr J. Westrop for carrying out some of the experimental work. The work was supported by a grant from the British Empire Cancer Campaign.

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Determination of the Arginase Activities of Homogenates of Liver and Mammary Gland: Effects of pH and Substrate Concentration and Especially of Activation by Divalent Metal Ions

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(Received 4 February 1948)

In our previous studies of the arginase activities of certain tissues of the rat (Folley & Greenbaum, 1946, 1947a) we used reaction periods of 30 min. and 20 hr. for homogenates of liver and mammary gland respectively, and assessed the activities of the homogenates in terms of our 30 min. or 20 hr. arginase units from enzyme calibration curves. Arginase undergoes inactivation at alkaline pH values (Hunter & Morrell, 1933a), a factor particularly serious in methods involving long reaction periods. Nevertheless, we feel confident that our previous findings regarding changes in the arginase activity of the mammary gland under various conditions are valid, since all values were read from a calibration curve which not only was reproducible with different mammary-gland homogenates, but also agreed with a curve obtained for one of these homogenates in the presence of Co^{++} (see Fig. 1). The significance of the latter agreement is not lessened by our present belief (see later section) that the effect of Co^{++} under these conditions was mainly to inhibit reversible inactivation due to dissociation of the enzyme system.

However, the use of different units for liver and mammary-gland homogenates was hardly practicable for further studies involving comparison of arginase activities of these tissues in the same animal, since the comparison can only be made if the quantitative relationship between the two units is known, and while the highly active liver homogenates can be assayed by both methods, the high dilution necessary for the 20 hr. method favours dissociation of the

Fig. 1. Calibration curve (circles) for the determination of arginase in mammary-gland homogenates by the method of Folley & Greenbaum (1947a). The bottom curve (black dots) represents the arginase activities of various dilutions of an homogenate less active than the one used for the construction of the calibration curve; the top curve (triangles) represents the arginase activities of corresponding dilutions of the same homogenate in the presence of Co^{++} . The upper horizontal scale (20 hr. arginase units) refers only to the central curve, to which the other two sets of points have been fitted by appropriate adjustment of their horizontal scales, i.e. transformation of their abscissae into 20 hr. arginase units. It will be seen that the bottom curve gives a very satisfactory fit to the standard calibration curve, while the greater part of the top curve provides an extension (broken line) of it.