18. METABOLISM OF POLYCYCLIC COMPOUNDS

4. PRODUCTION OF A DIHYDROXY-1:2:5:6-DIBENZ-ANTHRACENE FROM 1:2:5:6-DIBENZANTHRACENE

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(Received 31 December 1940)

WHEN anthracene is fed to rats and rabbits different stereoisomeric forms of 1:2-dihydroxy-1:2-dihydroanthracene and their glycuronic acid derivatives are excreted in the urine [Boyland & Levi, 1935; 1936]. Extension of the work to the metabolism of the carcinogenic compound 1:2:5:6-dibenzanthracene in rabbits has led to the isolation of a phenol, dihydroxy-1:2:5:6-dibenzanthracene by Boyland & Levi [1938]. The same compound was later obtained by Dobriner *et al.* [1939]. No positive evidence of the presence of hydrogenated derivatives with asymmetric carbon atoms corresponding to those produced from anthracene has been found, although such compounds might be excreted and later decomposed to give the phenol.

EXPERIMENTAL

Rabbits were fed on the following diet:

Wholemeal flour	35 parts
Wheat germ	30 "
Dried milk	10 "
Lard containing 0.8 % of 1:2:5:6-dibenzanthracene	20 "
Cod liver oil	2 "
NaCl	2 "
Dried yeast	1 part

The rabbits which were maintained on this diet for many months died generally with cirrhosis of the liver, similar to that described as resulting from injection of 1:2:5:6-dibenzanthracene into rabbits [Polson, 1936; Claude, 1937; Burrows & Boyland, 1938]. In some experiments in which the same 1:2:5:6-dibenzanthracene diet was fed to rats no cirrhosis was produced but the animals died in 6-10 weeks. With both species proteinuria frequently developed during the experiments.

The animals were kept in metabolism cages and the urine was collected daily, filtered through kieselguhr and stored at 0-5°. The urine was fluorescent and the isolation of products was followed by continuous examination of extracts in ultraviolet light.

Examination of sulphur compounds

The partition of the sulphur present in the urine was determined as described by Folin [1905] and the results obtained with two rabbits to which 1:2:5:6-dibenzanthracene was administered are given in Table 1. The significance of the

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results was calculated by the method of 't' [Fisher, 1936]. In both cases there is an increase in the proportion of ethereal sulphate present, indicating that the dibenzanthracene may be partly excreted as a sulphuric ester of a hydroxy derivative.

In one case (rabbit B) there was a considerable increase in the amount of neutral sulphur particularly after the rabbit had been on the dibenzanthracene

Table 1. *The effect of administration of 1:2:5:6-dibenzanthracene to rabbits on the sulphur partition in the urine*

Rabbit A. 7 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene				Rabbit B. 40 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene			
Percentage of total sulphur				Percentage of total sulphur			
Day	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Day	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	51.8	6.2	42.0	1	71.8	4.8	23.4
2	49.5	5.5	45.0	4	74.7	5.6	19.7
3	44.7	7.0	48.3	8	68.3	11.8	19.9
6	41.9	2.6	55.5	11	69.9	5.6	24.5
7	42.8	3.4	53.8	16	79.6	6.0	14.4
				40	69.5	7.5	23.0
13	63.5	7.8	29.7	42	60.1	10.2	29.7
14	50.5	3.2	46.3	46	66.5	5.8	27.7
15	44.5	9.1	46.4	47	5.8	11.2	30.4
27	53.0	11.0	36.0	49	74.5	4.5	21.0
35	53.8	13.2	33.0	53	60.5	6.2	33.3
57	34.7	14.6	50.7	103	28.0	8.0	64.0
63	50.7	7.6	41.7	109	31.6	8.5	54.5
78	44.1	3.8	52.1	118	37.7	8.1	54.2
Mean on normal diet		4.9	48.9			6.9	20.6
Mean on dibenzanthracene diet		8.8	42.1			8.5	37.2
Probability 'P' that difference in means is real		0.05	0.2			0.2	0.02

Table 2. *The effect of administration of 3:4:5:6-dibenzcarbazole to rabbits on the sulphur partition in the urine*

Rabbit C. 13 days on normal diet, then on diet containing 0.16% 3:4:5:6-dibenzcarbazole

Percentage of total sulphur			
Day	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	58.0	4.7	37.3
2	53.7	6.6	39.3
6	47.4	5.8	46.8
7	40.4	6.1	53.5
13	53.1	7.8	39.1
14	34.9	12.8	52.3
15	19.8	12.8	67.4
20	46.7	11.6	41.7
26	28.4	12.1	59.5
33	30.9	17.7	51.4
42	47.1	8.7	44.2
61	31.4	26.8	41.8
74	32.1	12.9	55.0
Mean on normal diet		6.2	43.2
Mean on dibenzcarbazole diet		12.8	51.7
Probability 'P' that increase is real		0.05	0.05

diet for 50 days. It seems probable that this increase in neutral sulphur is due to proteinuria following hepatic and renal injury. No mercapturic acid was isolated whereas if the increased neutral sulphur had been due to dibenzanthracene mercapturic acid, large amounts of this compound would have been present and its isolation should not have presented difficulties.

Some similar results with the carcinogenic 3:4:5:6-dibenzcarbazole are given in Table 2. Again there is a marked increase in the amount of ethereal sulphate and a small but definite increase in the neutral sulphur fraction. Other chemical investigations indicated that 3:4:5:6-dibenzcarbazole is excreted as a fluorescent hydroxy derivative. It is not possible to say whether in this case the increase in neutral sulphur is due to mercapturic acid or excreted protein, but rabbits to which 3:4:5:6-dibenzcarbazole was administered developed proteinuria.

Other determinations were made on the collected urine of 12 rats on normal diets and diets containing carcinogenic compounds. On adding 1:2:5:6-dibenzanthracene to the diet the ethereal sulphate rose slightly, from 8.1 to 9.6 %, and the neutral sulphur from 24.1 to 26.7 % of the total sulphur in the urine. The addition of 3:4:5:6-dibenzcarbazole to the rats' diet similarly increased the ethereal sulphate from 11.3 to 14.7 % and the neutral sulphur from 31.6 to 34.6 % of the total urine sulphur. These experiments indicate that these two carcinogenic substances are also excreted as sulphuric esters in rat's urine.

Isolation of dihydroxy-1:2:5:6-dibenzanthracene

This substance was obtained from the urine in two ways:

(a) The dried and powdered kieselguhr and precipitate were extracted in a Soxhlet apparatus with ether for some hours. The fluorescent ether extract was washed repeatedly with very dilute Na_2CO_3 solution until no further acidic material was present. This process removed much acid, the main part of which appeared to consist of stearic acid; M.P. after two crystallizations from aqueous alcohol 60–61°, equiv. wt. 280, 288 (stearic acid equivalent weight 284, M.P. 62.2°).

The residual ether was then extracted two or three times with dilute NaOH. The acidified extract gave a yellow flocculent precipitate of a phenol, which was collected and dried and recrystallized from acetic acid.

(b) The filtered urine in 2 l. lots was acidified, shaken with butyl alcohol in a large funnel, and allowed to stand for 12 hr. The emulsion remaining in the butyl alcohol layer was largely removed by filtration through kieselguhr. The butyl alcohol was then mixed with one volume of light petroleum, washed with water, and extracted with several small portions of dilute NaOH until the extracts were only faintly coloured. The combined alkaline extracts were washed with ether to remove dissolved butyl alcohol, first made acid with HCl, then alkaline with Na_2CO_3 , and the phenolic material extracted with ether. It was re-extracted with NaOH solution, precipitated with HCl, filtered, dried and recrystallized from acetic acid. It was identical with that obtained from the kieselguhr. From the two sources about 10 mg. were isolated from each litre of urine.

Dihydroxy-1:2:5:6-dibenzanthracene crystallized in yellow needles which darken on exposure to light; M.P. 340–350° with decomposition; soluble in alcohol, acetone and ether to give violet-fluorescent solutions; almost insoluble in CHCl_3 and benzene. It was not extracted from ethereal solution by Na_2CO_3 solution. The solution in NaOH was yellow with a bright green fluorescence and

was fairly stable in air. The aqueous and alcoholic solutions gave no reaction with FeCl_3 . A 1% solution of the substance in dioxane ($c=1\%$) showed no optical activity. Analysis (Schoeller), found: C, 84.2%; H, 5.6%. $\text{C}_{22}\text{H}_{14}\text{O}_2$ requires: C, 85.2%; H, 5.5%.

This phenol could be obtained by alkaline hydrolysis of the purified diacetyl derivative and was more conveniently purified and obtained in better yield in this way.

Diacetoxy-1:2:5:6-dibenzanthracene. The dihydroxy-1:2:5:6-dibenzanthracene obtained from urine was dissolved in one volume of pyridine and the solution boiled with ten volumes acetic anhydride for 5 min. The solution was hydrolysed with water and the product crystallized from acetic acid in colourless plates, m.p. 291° . It was slightly soluble in ethyl alcohol and the solution had a blue-violet fluorescence.

Analysis (Weiler), found: C, 78.0%; H, 4.5%. $\text{C}_{26}\text{H}_{18}\text{O}_4$ requires: C, 78.1%; H, 4.6%.

Diacetoxy-1:2:5:6-dibenzanthraquinone. Diacetoxy-1:2:5:6-dibenzanthracene on warming with CrO_3 in acetic acid gave a quinone which crystallized from hot acetic acid in yellow needles, m.p. $294\text{--}296^\circ$. It was soluble in alcohol and dissolved slowly in alkaline $\text{Na}_2\text{S}_2\text{O}_4$ to give a clear orange solution. 1:2:5:6-Dibenz-9:10-anthraquinone gives a red solution in alkaline $\text{Na}_2\text{S}_2\text{O}_4$. Analysis (Weiler), found: C, 78.3%; H, 4.0%. $\text{C}_{26}\text{H}_{16}\text{O}_4$ requires: C, 78.6%; H, 3.8%.

Dimethoxy-1:2:5:6-dibenzanthracene. Dihydroxy-1:2:5:6-dibenzanthracene treated with dimethyl sulphate in NaOH solution gave a methyl ether which crystallized from hot acetic acid in pale brown needles, m.p. $244\text{--}245^\circ$. It gave violet-fluorescent solutions in acetone and alcohol. Analysis (Schoeller), found: C, 85.0%; H, 5.7%. $\text{C}_{24}\text{H}_{18}\text{O}_2$ requires: C, 85.3%; H, 5.3%.

Dimethoxy-1:2:5:6-dibenzanthraquinone. Oxidation of the dimethyl ether with CrO_3 in acetic acid gave poor yields of the quinone, which crystallized from ethyl alcohol in red plates, m.p. 264° . Analysis (Schoeller), found: C, 77.2%; H, 4.40%. $\text{C}_{24}\text{H}_{16}\text{O}_4$ requires: C, 78.0%; H, 4.32%. The solution in NaOH with $\text{Na}_2\text{S}_2\text{O}_4$ was clear yellow.

Dihydroxy-1:2:5:6-dibenzanthraquinone. The diacetyl derivative of the quinone when warmed with 2N NaOH gave a deep blue solution from which a nearly black Na salt separated on standing. The blue solution turned orange on being warmed with Zn dust or $\text{Na}_2\text{S}_2\text{O}_4$.

The hydroxyquinone was prepared by warming the diacetyl derivative with alcoholic alkali for a few minutes. The deep blue solution was poured into dilute acid, when a red gelatinous precipitate separated. This was collected, dried and recrystallized from glacial acetic acid, when it formed deep red needles, m.p. 350° . Analysis (Schoeller), found: C, 77.0%; H, 3.8%. $\text{C}_{22}\text{H}_{12}\text{O}_4$ requires: C, 77.7%; H, 3.5%.

On heating with boracetic anhydride in acetic anhydride the dihydroxyquinone did not form a boroacetate, but the original quinone acetate was recovered. The substance was readily methylated with dimethyl sulphate in NaOH. Its blue alkaline solution was changed to green by the addition of diazotized sulphanilic acid.

The quinone (100 mg.) was oxidized with acid KMnO_4 under conditions identical with those described for the oxidation of 1:2:5:6-dibenzanthraquinone to anthraquinone tetracarboxylic acid [Cook, 1931]. No anthraquinone tetracarboxylic acid could be isolated, whereas had the hydroxyl groups been situated in the side rings this product should have been obtained.

Absorption spectra

The absorption spectra were measured by means of a Hilger medium quartz spectrograph and a Spekker photometer [cf. Mayneord & Roe, 1935]. Alcohol was used as solvent throughout. 1:5-Dihydroxyanthraquinone was sparingly soluble in alcohol and gave varying results when the suspension was warmed to hasten solution. The solution finally used was obtained by shaking for some hours in the dark.

A comparison of the spectrum of the phenol (dihydroxy-1:2:5:6-dibenzanthracene, Fig. 1) with that of 1:2:5:6-dibenzanthracene [Mayneord & Roe, 1935] indicates clearly that it is a 1:2:5:6-dibenzanthracene derivative. The spectrum is shifted 50–100 Å. towards the visible compared with 1:2:5:6-dibenzanthracene; its absorption intensity is diminished, and its long-wave band system is simpler in form.

Substitution by a methyl group in a side-ring of 1:2:5:6-dibenzanthracene, e.g. 2'- or 3'-methyl-1:2:5:6-dibenzanthracene, has very little effect on the absorption spectrum. The resulting spectrum shows slightly lower band-intensities and 2–20 Å. band-shifts towards the visible compared with that of 1:2:5:6-dibenzanthracene [E. Roe, unpublished results]. No data are available on the effect of the substitution by hydroxyl groups in the side-rings, and although the spectrographic evidence below suggests that in the phenol the hydroxyl groups are substituted in the anthracene nucleus, it does not eliminate the possibility of substitution in the side-rings.

In order to obtain further information as to the position of the hydroxyl groups in the phenol, the absorption spectrum of its quinone has been examined and compared with that of 1:2:5:6-dibenzanthraquinone (Fig. 1). The main absorption band of the latter at 2962 Å. is shifted to 3042 Å. in the dihydroxy-derivative and its 2375 Å. band is similarly shifted to 2484 Å. There are greater differences in the long-wave region however, the band at 3900 Å. in 1:2:5:6-dibenzanthraquinone being replaced by one at 4535 Å. in its dihydroxy-derivative.

Fig. 1. •—• Dihydroxy-1:2:5:6-dibenzanthracene.
○—○ Dihydroxy-1:2:5:6-dibenzanthraquinone. — 1:2:5:6-Dibenzanthraquinone.

These spectra may be compared with those of anthraquinone and its 2:6- and 1:5-dihydroxy-derivatives (Fig. 2). The spectrum of 2:6-dihydroxyanthraquinone is similar in form to that of anthraquinone and is shifted 200–300 Å. towards the visible. 1:5-Dihydroxyanthraquinone presents a different type of spectrum. The short-wave bands are shifted slightly towards the visible compared with anthraquinone, but the band at 3230 Å. in the latter is replaced by a band at 4230 Å. in the 1:5-dihydroxy-compound, a difference of 1000 Å.

Fig. 2. — Anthraquinone. •—• 1:5-Dihydroxyanthraquinone.
○—○ 2:6-Dihydroxyanthraquinone.

Table 3. Wave-lengths of maxima, with extinction coefficients

λ = wave-length in Å.; $\epsilon = \log \frac{I_0}{I} / l \cdot c$ c = concentration in g.-mol. per l. l = cm.			
λ	$\log \epsilon$	λ	$\log \epsilon$
Dihydroxy-1:2:5:6-dibenzanthracene.		9:10-Anthraquinone	
2298	4.64	2514	4.69
2823	4.49	2711	4.19
2928	4.81	3230	3.69
3045	4.95		
3289	4.04	2:6-Dihydroxy-9:10-anthraquinone	
3430	4.04	2190	4.41
3590	4.12	2733	4.54
3848	3.34	3004	4.33
4070	3.35	3475	3.90
Dihydroxy-1:2:5:6-dibenz-9:10-anthraquinone		1:5-Dihydroxy-9:10-anthraquinone	
2240	4.59	2253	4.97
2484	4.59	2515	4.30
3042	4.49	2768	4.00
4535	3.83	2860	3.99
1:2:5:6-Dibenz-9:10-anthraquinone		4230	4.00
2375	4.58		
2962	4.81		
3328	3.79		
3900	3.81		

This change is similar to that occurring in the spectrum of the dihydroxy-1:2:5:6-dibenzanthraquinone derived from the phenol when compared with its parent 1:2:5:6-dibenzanthraquinone and it suggests that the hydroxyl groups in the phenol may occupy the 1:5-positions of the anthracene nucleus, i.e. that the phenol isolated from rabbit urine is 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

Tests for carcinogenic activity

The dihydroxy-1:2:5:6-dibenzanthracene was not sufficiently soluble in benzene to be applied by painting to mouse skin in that solvent. Experiments were made with an acetone solution of the substance (0.1 %), with which the backs of 10 mice were painted twice weekly. None of the mice developed tumours although one mouse survived the treatment for 16 months and another for 18½ months. In another experiment 10 mice were injected with 2 doses each of 1 mg. of the phenol suspended in olive oil, with an interval of one week between injections. All the mice died without tumours between the 51st and 119th day following the injection. This is in agreement with the findings of Dobriner *et al.* [1939].

DISCUSSION

It has unfortunately been necessary to interrupt this work before the suggested structure of the dihydroxydibenzanthracene could be tested by synthesis and also before making a complete examination of the excretion of dibenzanthracene in the rat.

Rats and rabbits produce different products from anthracene [Boyland & Levi, 1935; 1936] and also react differently to 1:2:5:6-dibenzanthracene. The injection of 1:2:5:6-dibenzanthracene into rats readily produces tumours at the site of injection while similar injections into rabbits do not [Burrows & Boyland, 1938]. It is possible that this difference in response is due to a difference in the metabolism of the carcinogen analogous to that found in anthracene metabolism.

The non-carcinogenic phenol isolated from the urine of rabbits which were fed with the carcinogenic 1:2:5:6-dibenzanthracene may have been present as a tetrahydroxytetrahydro-1:2:5:6-dibenzanthracene or the corresponding glucuronide if the metabolism of dibenzanthracene is similar to that of anthracene. In that case the dibenzanthracene derivative must be less stable than the corresponding dihydroxydihydroanthracene which was converted into 1-hydroxyanthracene on gentle heating with dilute acid.

The positions of the hydroxyl groups in the molecule of the phenol cannot definitely be decided. The dihydroxydibenzanthracene even in alkaline solution was stable to air and did not give colour reactions with ferric chloride. It is therefore improbable that the hydroxyl groups are attached to adjacent carbon atoms or to carbon atoms in the *p*-positions in a benzene ring.

The fact that oxidation with KMnO_4 did not yield anthraquinone tetracarboxylic acid indicates that the hydroxyl groups are not in the side rings of the dibenzanthracene molecule. Cason & Fieser [1940] have synthesized 4':8'-dihydroxy-1:2:5:6-dibenzanthracene which they describe as being probably identical with the substance excreted when dibenzanthracene is injected into rats and mice. This differs from the product excreted by rabbits which Cason & Fieser [1940] tentatively suggest is 3':7'-dihydroxy-1:2:5:6-dibenzanthracene.

The formation of the diacetoxy-, dimethoxy- and dihydroxy-dibenzanthraquinone excludes the 9 and 10 positions. The non-formation of a boracetic ether

suggests that neither of the hydroxyl groups is in the *peri* position to the quinone groups. The ease with which the dihydroxyquinone reacts with dimethyl sulphate is also in agreement with the hypothesis that the hydroxyl groups are in 3:7 positions. On the other hand the spectrographic evidence suggests that the compound might be 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

Possible formulae for the excretion product therefore include 3:7-dihydroxy-1:2:5:6-dibenzanthracene (I), 4:8-dihydroxy-1:2:5:6-dibenzanthracene (II) and 3:8-dihydroxy-1:2:5:6-dibenzanthracene (III).

SUMMARY

1. The administration of 1:2:5:6-dibenzanthracene or 3:4:5:6-dibenzcarbazole to rats or rabbits causes an increase in the excretion of ethereal sulphate in the urine.

2. A phenolic derivative, dihydroxy-1:2:5:6-dibenzanthracene was isolated from the urine of rabbits fed on a diet containing 1:2:5:6-dibenzanthracene. This phenol has been characterized and several derivatives prepared.

We are indebted to the British Empire Cancer Campaign for their support of this work, and to the Sir Halley Stewart Trust and the International Cancer Research Foundation for generous grants.

We wish to thank Messrs Hoffman La Roche for a gift of 3:4:5:6-dibenzcarbazole.

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