CXCII. CHANGES IN THE LIVERS OF MICE AFTER ADMINISTRATION OF 3:4:5:6-DIBENZCARBAZOLE

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THE known carcinogenic hydrocarbons produce primary tumours at the site of application [cf. Cook *et al.* 1937] and one of them at any rate (1:2:5:6-dibenzanthracene) increases the incidence of lung cancer in those strains of mice in which this condition occurs spontaneously [Lynch, 1935; Andervont, 1935]. The feeding [Sasaki & Yoshida, 1935] or injection [Shear, 1937] of 4′-amino-2:3′-azotoluene induces malignant changes in the livers of rats and mice. This compound, however, does not have any marked carcinogenic action at the site of administration. 3:4:5:6-Dibenzcarbazole produces sarcomata as a result of subcutaneous injection into rats and epitheliomata and bile duct hypertrophy resembling cholangiomata when it is painted twice weekly on the skin of mice [Boyland & Brues, 1937]. Strong *et al.* [1938] found that the injection of 3:4:5:6-dibenzcarbazole into mice of the C.B.A. strain, in which hepatoma occurs spontaneously, increased the incidence of these tumours.

As 3:4:5:6-dibenzcarbazole produces bile duct proliferation and neoplastic changes in the livers an attempt has been made to correlate the histological appearances with certain chemical changes. This compound is much more toxic to mice than carcinogenic hydrocarbons such as 1:2:5:6-dibenzanthracene or methylcholanthrene.

Methods. Mixed stock mice previously fed on Purina Fox Chow for a week received a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole dissolved in olive oil. Mice weighing less than 16 g. were not used as it was found that the compound was more toxic to young mice. During the experiment the diet consisted entirely of Fox Chow. At varying intervals after the injection mice were weighed, killed and their livers removed. A small portion of the liver was taken for histological section, and the remainder was investigated chemically. In some cases a small piece was also taken for estimation of the glycolysis and respiration in the Warburg apparatus.

The liver tissue, after weighing, was ground in a centrifuge tube with 3 vol. of 5 % trichloroacetic acid, the suspension centrifuged and an aliquot portion of the supernatant fluid removed for determination of ascorbic acid and glutathione as described by Mawson [1935]; the ascorbic acid was first titrated with phenol-indo-2:6:dichlorophenol and then the glutathione with iodine. The trichloroacetic acid precipitate was saponified with KOH on a boiling water bath and the "fat" estimated by the Liebermann method as described by Leathes & Raper [1925]. According to Dulière and Minne [1937] cholesterol is completely removed from blood serum by precipitation with trichloroacetic acid and we have found that estimations of the fat content on the trichloroacetic acid precipitate of liver give the same values as estimations on fresh tissue. The cholesterol in the

"fat" was determined by a modification of the method of Schoenheimer & Sperry [1934] for blood; the material was dissolved in 5 ml. acetone-alcohol (1:1) and 2 ml. 0·5 % digitonin in 50 % alcohol were added. The solution was allowed to stand overnight and next day the precipitate was centrifuged and washed once with acetone-ether (1 : 2) and twice with ether. The last traces of ether were removed in an oven at 40° and the precipitate dissolved in 4 ml. glacial acetic acid. 8 ml. acetic anhydride and 0·4 ml. conc. H_2SO_4 were added and the colour which developed was compared in a colorimeter with that of a cholesterol standard, using a red screen.

Results

Series 1. In the first experiment 0·5 mg. 3:4:5:6-dibenzcarbazole was given to each mouse, and there was a considerable mortality during the week following the injection. Of a series of 38 mice, 10 were alive on the 3rd day after injection; 4 of these were killed and only 3 of the remaining 6 were alive on the 5th day. Many of the livers were very pale and appeared fatty. The most striking chemical change was in the glutathione (Fig. 1); the amount in many cases was definitely

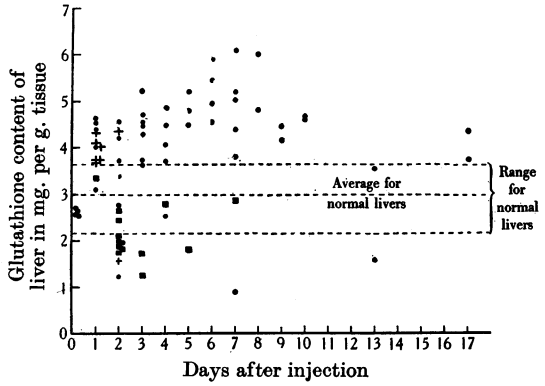

Fig. 1. The glutathione content of livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole. • Represents liver of normal histological appearance. ■ Represents liver with advanced necrosis. + Represents liver with early necrosis.

increased and in 3 mice was twice the normal average. This average (2·91 mg. per g. with a standard deviation ±0·37) was obtained from 30 untreated animals. Eight control mice injected with 0·5 ml. olive oil and killed 3–10 days afterwards had an average liver glutathione of 2·68 with a standard deviation ±0·23. In all the 4 mice killed 3–4 hr. after injection with 3:4:5:6-dibenzcarbazole the amount was within the normal range. At the end of 24 hr. the rise was quite definite and the highest values occurred between the 3rd and 8th days. This rise in glutathione, however, did not occur invariably. Among 16 mice killed 48 hr. after injection 4 were within the normal range and 8 were below it. But 31 out of 40 mice killed after 48 hr. showed a glutathione content above the highest limit of the normal range.

The ascorbic acid content of these livers showed no significant change. There was great variation among apparently normal mice (0·152–0·465 mg. per g.) and 59 out of 67 treated animals (86 %) were within this range. In all cases where the glutathione was very low the ascorbic acid was below the normal average. Fat and cholesterol estimations were not carried out in this experiment.

Histological examination of paraffin sections (special methods for staining fats were not used) suggested that many of the livers were either very fatty or contained glycogen, in agreement with the pale colour seen with the naked eye. However, fat or glycogen appeared to have been present in sections of the livers of untreated mice which had been fed on Fox Chow and, as it is impossible to estimate the amount of fat histologically, no conclusion could be drawn. No proliferation of the bile ducts was observed in these mice of which none lived more than 17 days. The most striking feature observed histologically was the differentiation of the liver lobule into an inner zone of well stained cells surrounding the central vein and an outer zone of large degenerate cells which appeared to have contained fat or glycogen. This zoning was observed in less than one-third of the mice and in some of these was very slight. The glutathione content of these has been indicated in Fig. 1, which shows that most of the slightly necrotic livers occurred 24 hr. after the injections and the glutathione in these was above the normal limit. Seven out of 13 livers showing toxic necrosis and degeneration were in mice which had been injected 48 hr. previously, and all of these (except one at 24 hr.) contained less than the normal average of glutathione, and the majority (8 out of 13) less than the lowest normal limit. The most advanced necrosis was seen in two livers at the 4th and 7th days.

None of the livers of mice killed 8 days or more after injection showed necrosis or hepatitis indicating that recovery and regeneration occurred. Many of these livers showed signs of regeneration and growth. With the completion of recovery the glutathione content returned almost to the normal value.

Determinations of the respiration and glycolysis (Table I) show that the four livers with necrotic areas on which these determinations were made had low values for respiration, with Q_{O_2} ranging from $-2{\cdot}3$ to $-7{\cdot}6$ instead of the

Table I. *Livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole*

Time after injection days	Glutathione mg. per g.	Ascorbic acid mg. per g.	Metabolism: Respiration Q_{O_2}	Metabolism: Glycolysis $Q_L^{N_2}$	Histological appearance
2	1·22	0·135	− 3·4	+1·4	—
2	3·73	0·310	−14·3	+4·1	—
2	2·44	0·152	− 7·6	+2·0	Intense hepatitis
2	2·02	0·191	− 3·6	+1·6	,,
3	1·23	0·200	− 2·3	+1·3	,,
3	4·47	0·428	−10·8	—	—
3	4·52	0·333	− 9·1	+3·4	—
4	3·70	0·258	− 9·7	—	—
6	4·97	0·285	− 7·0	+3·2	—
6	5·98	0·250	−10·8	+2·3	—
7	0·86	0·106	− 9·2	+6·1	—
7	6·08	0·330	− 5·0	+3·1	—
7	2·82	0·250	− 3·7	+1·3	Intense hepatitis
8	4·82	0·293	− 6·9	+2·5	—
9	4·17	0·292	− 8·6	+1·4	—
10	4·66	0·099	− 7·0	—	—
10	4·62	0·149	− 3·3	+3·9	—
13	1·59	0·038	− 3·2	—	—
13	3·52	0·460	−11·7	—	—
17	4·37	0·234	− 4·8	+3·8	—
17	3·72	0·148	− 2·3	—	—

normal values of -10 to -12. The metabolism of the other livers is on the whole low but irregular.

Series 2. The mortality in the first experiment (Fig. 1) was high; no mice were examined after 17 days, and therefore the chance of any development of

neoplastic changes in the liver was small; hence the dose was reduced to 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse (Fig. 2). Ascorbic acid, glutathione, fat and cholesterol were estimated on all livers in this experiment. Many died (of a series of 65 mice 28 (43 %) were alive 39 days after injection) but many lived for long periods and those which survived for 200 days appeared healthy. One mouse killed on the 176th day after injection had a spindle-celled sarcoma at the site of injection. The livers of many killed after the 40th day showed a nodular appearance and in others the lobes seemed to be swollen and often two or more lobes appeared fused.

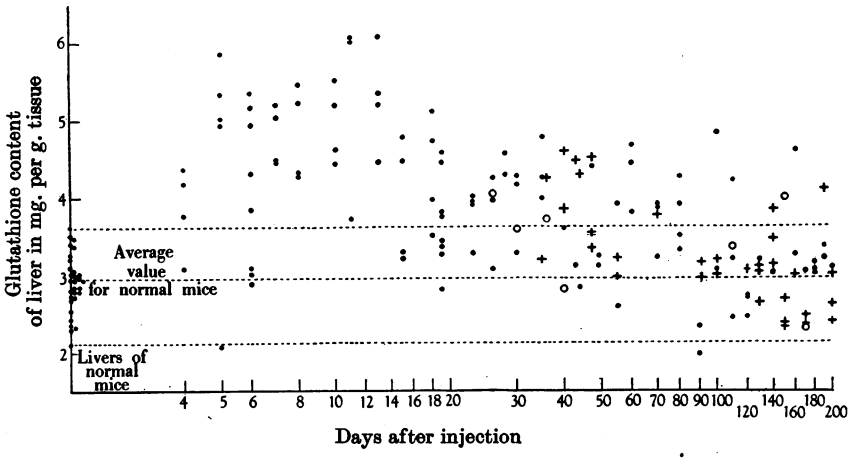

Fig. 2. The glutathione content of livers of mice injected with 3:4:5:6-dibenzcarbazole (12·5 mg. per kg. body wt.). • Represents liver with normal appearance of bile ducts. o Represents liver with early bile duct proliferation. + Represents liver with advanced bile duct proliferation.

The glutathione contents of the livers are shown in Fig. 2. Again a very distinct increase was observed which was much more consistent than in the earlier experiment. Only three values below the normal average occurred in the 40 days following injection and two of these were close to the normal average. The glutathione content was greatest during the first 20 days and then fell off gradually. Among 34 livers examined between the 120th and 200th days there were only four values above the normal range. The ascorbic acid content lay almost wholly within the wide normal range. The cholesterol and fat contents also remained approximately normal except for a few isolated cases. The cholesterol content varied from 0·216 to 0·645 % except for two livers with 0·169 and 1·075 % cholesterol. The liver fat content ranged from 2·1 to 8·6 % with two exceptions of 1·6 and 11·2 %. The variations in fat and cholesterol could not be correlated with histological appearance. The hepatitis and necrosis which were so striking in the first series were seen in only 7 mice. The instances of early bile duct proliferation and advanced bile duct hypertrophy (the distinction between these two conditions is quite arbitrary) are indicated in Fig. 2. The advanced biliary hypertrophy resembles cholangioma but as transplantation experiments were not performed it is not possible to decide whether this type of growth is malignant or not. No cases of advanced hypertrophy occurred before the 35th day and 33 of 83 mice (40 %) killed after this time showed definite bile duct proliferation. One mouse on the 26th day and another on the

30th day showed indications of the development of this condition. Cholangiomatous growth was observed in livers both with normal and with increased glutathione.

Table II. *Livers of mice injected with 3:4:5:6-dibenzcarbazole (12·5 mg. per kg. body weight)*

Time after injection days	Glutathione content mg. per g.	Ascorbic acid content mg. per g.	Metabolism Q_{O_2}	Metabolism Q_L^N	Histological appearance
10	5·18	0·37	− 8·3	4·9	—
90	1·98	0·51	−13·5	1·2	—
90	2·33	0·29	−13·8	3·5	—
163	2·90	0·56	− 8·9	1·5	—
163	3·40	0·42	−10·4	4·6	—
164	4·05	0·41	− 5·6	2·6	—
164	3·54	0·41	− 8·1	2·1	Advanced bile duct hypertrophy
164	3·54	0·37	− 7·7	4·4	—
172	3·35	0·25	−14·7	2·0	Advanced bile duct hypertrophy
172	3·55	0·31	− 9·0	2·1	,,
172	3·23	0·36	− 9·6	2·4	,,
176	2·72	0·30	− 7·8	1·1	,,
176	2·15	0·33	− 6·2	4·3	,,
176	4·50	0·17	− 6·8	2·7	,,
176	3·65	0·27	− 7·7	1·3	,,

Table III

Time after injection days	Wt. before injection g.	Wt. after injection g.	Wt. of liver g.	Glutathione mg. per g.	Ascorbic acid mg. per g.	Fat %	Cholesterol %
Livers of mice injected with 1 mg. methylcholanthrene							
4	23	17·0	0·89	2·16	0·190	3·4	0·37
4	27	19·5	1·12	2·60	0·331	3·8	0·31
4	27	19·5	1·28	2·27	0·336	4·1	0·30
4	23	20·0	1·32	2·60	0·315	4·4	0·27
8	27	21·0	1·18	2·23	0·304	3·6	0·38
8	25	20·5	1·00	2·79	0·325	4·8	0·35
8	18	17·5	1·02	3·29	0·375	3·6	0·28
8	22	17·5	1·10	3·30	0·358	3·5	0·34
12	24	17·5	0·90	2·77	0·272	3·1	0·32
12	21	16·5	0·78	2·40	0·281	3·1	0·40
12	26	22·0	1·05	2·88	0·380	4·1	0·33
12	18	16·5	0·98	3·77	0·370	3·8	0·31
16	20	28·0	2·30	3·37	0·422	2·9	0·31
16	21	27·5	2·16	2·72	0·411	3·0	0·28
16	19	22·0	1·73	3·00	0·400	3·2	0·38
16	16	21·5	1·85	3·80	0·450	4·0	0·32
20	—	26·0	1·66	3·12	0·406	4·0	0·27
20	18	20·0	1·34	2·81	0·433	3·9	0·26
20	—	16·0	0·84	2·50	0·282	3·6	0·32
20	—	20·0	1·46	2·82	0·502	3·8	0·28
Livers of mice injected with 2 mg. 1:2:5:6-dibenzanthracene							
3	—	—	—	3·00	0·217	2·9	—
4	—	—	—	2·78	0·324	3·4	—
4	—	—	—	3·47	0·291	3·5	—
6	—	—	—	2·50	0·272	3·6	—
6	—	—	—	2·58	0·299	3·0	—
7	—	—	—	1·76	0·328	3·5	—
7	—	—	—	3·63	0·230	5·0	—
10	—	—	—	3·37	0·239	3·5	—

The metabolisms of the livers of some of the mice were determined and the results obtained are given in Table II. The metabolism of livers with advanced biliary hypertrophy is of the same order as that of normal livers.

Series 3. Eight mice which were injected intraperitoneally with 2 mg. 1:2:5:6-dibenzanthracene in olive oil and killed 3–10 days afterwards showed no significant changes in the glutathione, ascorbic acid or fat content of the liver (cf. Table III). No histological examination was carried out.

Twenty mice were injected intraperitoneally with 1 mg. methylcholanthrene dissolved in 0·25 ml. lard per 20 g. mouse and killed at intervals up to 20 days after the injection. The livers of these animals appeared normal except for numerous adhesions. The analytical results obtained on these livers are shown in Table III: no significant changes were observed and histological examination of all the livers revealed no abnormalities.

Discussion

Considerable changes in the livers of mice may occur as the result of a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole (0·25 or 0·5 mg.). Boyland & Brues [1937] found that rats receiving twice weekly subcutaneous injections of 1 mg. of this compound lived for a long time and showed no liver lesions although spindle-celled sarcomata developed at the site of injection, while mice receiving this treatment died after one or two injections. In our own experiments the mortality was also high in mice which were given a single dose of 0·5 mg. 3:4:5:6-dibenzcarbazole and striking histological changes (hepatitis and necrosis) were observed in the livers of many of those killed during 7 days following the injection. It would appear that 3:4:5:6-dibenzcarbazole is more toxic to mice than to rats and this may possibly be associated with its effect on the liver in the mouse. With 0·25 mg. of the compound the mortality was much less and few cases of hepatitis were observed.

The first definite proliferation of bile ducts was observed 35 days after injection of 0·25 mg. although the first indication of such development was seen at the 26th day. These times agree very well with those at which Boyland & Brues observed similar biliary proliferation in mice painted twice weekly with the same compound.

The glutathione content of the liver had increased 24 hr. after injection and was at its maximum during the first 20 days, after which it decreased. Since the ascorbic acid, fat and cholesterol contents remained approximately normal we consider that this is a true increase in glutathione and is not due to a decrease in the water content of the liver. The glutathione content was at its maximum before any neoplastic change was observed (Fig. 2). Some of the "cholangiomata" were seen in livers with an increased glutathione content but most of them occurred when the glutathione had returned to normal.

In the first experiment the livers with highest glutathione content were found after the period when the histological changes were most marked. High glutathione content was often present in those livers in which regeneration was taking place. In about 10 days when the regeneration was complete the glutathione content fell again. In the second experiment, in which the dose of 3:4:5:6-dibenzcarbazole was less, very high values for glutathione content were found during the first 20 days and during this time recovery from the injection with regeneration of cells was probably taking place. Although in many cases the livers were greatly enlarged it was not possible to correlate liver weight with glutathione content. The results indicate that the 3:4:5:6-dibenzcarbazole has

a toxic action which is followed by regeneration accompanied by an increase in glutathione, and at a later stage bile duct hypertrophy takes place.

The decrease in glutathione content which was observed (Fig. 1) in certain mice receiving 0·5 mg. 3:4:5:6-dibenzcarbazole was accompanied by a decrease in ascorbic acid and both the macroscopic appearance of the livers and the study of histological sections suggest that this may have been due in part at least to an accumulation of fat.

1:2:5:6-Dibenzanthracene, which produces hepatoma in mice of the C_3H strain [Andervont, 1937] but has not been shown to produce proliferation of bile ducts, and methylcholanthrene, which is not known to produce either of these changes, did not affect the glutathione, ascorbic acid or fat content of the livers of mice.

Summary

A single intraperitoneal injection of 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse caused an increase in the glutathione content of the liver which was maximal during the first 20 days. This preceded a considerable proliferation of bile ducts which occurred in 40 % of the mice examined between the 35th and 200th days. When 0·5 mg. was given there was a similar increase in glutathione content in the majority of the mice but in a few there was a decrease which in most cases was accompanied by a partial necrosis of the liver lobules. This increase in glutathione was not observed in mice injected with 1:2:5:6-dibenzanthracene or with methylcholanthrene. No change in the ascorbic acid or cholesterol of the liver occurred with any of these carcinogenic compounds.

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