The Effect of Carcinogenic Compounds on the Ascorbic Acid Content of the Liver in Mice and Rats

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The experiments recorded below are a continuation of an earlier investigation (8) on the effect of aromatic compounds, carcinogenic and non-carcinogenic, upon the ascorbic acid content of the liver in mice.

In the present series, four compounds were used, namely *p*-dimethylamino-azobenzene (*p*-DA), 2-acetamidofluorene (AAF), 7-hydroxy-2-acetamidofluorene (7-OH-AAF) (4) and azobenzene (AB). The experiments fall into two groups: (a) *Short term (up to 13 days) injection experiments.* Subcutaneous and intraperitoneal injections in mice (CBA) and intraperitoneal injections in rats (Wistar and black-and-white) of AAF and 7-OH-AAF only. (b) *Long-term (up to 460 days) feeding experiments* which give time for the development of tumors. The four compounds were added to various diets given to Wistar rats which were used in other investigations as well (3, 5).

METHODS

The liver was removed at once, and the whole organ, in the case of a mouse, or one or more portions of similar weight (about 1 gm.) from the liver of a rat, homogenized by the method of Potter and Elvehjem (9) in 10 ml. 3 per cent metaphosphoric acid, and centrifuged, and the process of mixture and centrifugation repeated twice, using 30 ml. metaphosphoric acid in all. The extract was titrated with 2.6-dichlorophenolindophenol. The substance in mouse liver which reacts with this dye, and the increment of it induced by carcinogens, is destroyed by vegetable marrow juice (8) and may therefore be assumed to be ascorbic acid.

I. Injection Experiments on Mice and Rats

The animals used in these experiments received the ordinary mixed diet given to the laboratory stock. The water content of the livers was not estimated.

(a) *Mice.*—Fifty-two mice (CBA males and females) received 2 intraperitoneal or subcutaneous injections of AAF or 7-OH-AAF (10 mgm. in 0.2 ml. arachis oil) or of 0.2 ml. of arachis oil only over a period of 4 to 6 days; they were killed between the sixth and 13th day after the first injection (Table I). Controls receiving arachis oil are essential because this oil, and sesame oil, alone, increase the ratio of liver weight to body weight in male mice, *e.g.* from 4.8 to 5.4 per cent (7). The figures for the concentration of ascorbic acid in the liver show the wide range of variation found in mice (7), but there is in the male CBA mice a statistically significant increase of 23 per cent following injection of 2-acetamidofluorene, while the females show no significant change. The ratio of liver weight to body weight shows no distinct differences in the various series. Intraperitoneal injection of 7-OH-AAF produced no increase in the ascorbic acid in either sex.

(b) *Rats.*—Sixty-three rats (Black-and-White, Wistar, males and females) received 2 intraperitoneal injections for from 3 to 5 days of AAF or 7-OH-AAF (30 mgm. in 1 ml. of arachis oil) or of 1 ml. arachis oil only: they were killed between the fifth and eighth day after the first injection (Table II).

The black-and-white rats showed a mean increase in concentration of ascorbic acid in the liver after injection of AAF which was of the same degree (34 to 35 per cent) in both sexes, and was therefore greater than that seen in the CBA mice. The 7-OH compound produced only a very slight increase.

Data on the Wistar rats reveal a very wide range of variation. Injection of AAF produced a mean increase in both sexes of only about 10 per cent, which is no greater than that seen in the black-and-white rats after injection of the 7-OH compound, whereas the mean figures obtained in 4 rats with this latter compound were practically the same as those from the controls, 269 and 267 in males; 232 and 235 in females.

The ratio of liver weight to body weight shows no constant differences in the various series. Hence the results of the injection experiments show: (a) that the black-and-white rats respond to the action of AAF by an increased concentration of ascorbic acid in the liver much more than do Wistar rats;

TABLE I: INJECTION EXPERIMENTS IN CBA MICE WITH 2-ACETAMIDOFLUORENE

Experiment	Day of injection	Killed on day after first injection	Ascorbic acid in liver, μgm. per gm. wet weight — Male: Control S.	Control P.	A.A.F. S.	A.A.F. P.	7-OH A.A.F. P.	Female: Control S.	Control P.	A.A.F. S.	A.A.F. P.	7-OH A.A.F. P.
1	1,5	6	...	289	385	351		266	...	297	352	
		12	322	...	428	469		...	345	272	262	
	1,4	7	...	361	...	388		...				
2		9	348	...	394	...		...				
		10	...	296	...	370		...				
		13	351	...	...	...		298				
								351				
3	1,6	8	270	...	...	373		...	238	...	258	
		9	307	...	...	335		...	272	...	290	
4	1,5	6	...	226	...	370	260	...	292	...	351	278
5	1,6	6	...	226	...	301	220	...	217	...	315	226
		11	...	362	...	380	365	...	272	...	330	310
		Mean	340	297	402*	371†	283	305	284	299	304	271
			309		379			290		303		
			100		123			100		104		

* P 0.02 S = Subcutaneous injection
† P 0.001 P = Peritoneal injection.

(b) that the sexual difference in CBA mice, in that the males respond to AAF and the females do not, is absent in rats; and (c) that 7-OH-AAF is almost or quite inactive both in rats and in male mice.

II. FEEDING EXPERIMENTS ON WISTAR RATS

A semi-synthetic diet with and without the addition of *p*-DA, AAF or 7-OH-AAF was given, as follows:

		Calories, %
Starch	1,260	39.6
Sugar	630	18.6
Casein (fat & vitamin free)	342	12.7
Casein (commercial)	177	
Lard	420	29.1
Cod liver oil	7	...
Mineral salt mixture (Glaxo)	126	...

The diet was supplemented daily with 1 or 2 gm. of carrots or greens and about 1 gm. of "National Bread" (80 to 82 per cent extraction). The AAF, *p*-DA or 7-OH-AAF was added in amounts to give a final concentration of 0.07 per cent. Growth was about the same on the control diet and on those containing *p*-DA, AAF and 7-OH-AAF.

Another diet of rice with and without the addition of 6 per cent casein and of *p*-DA or AB (0.06 per cent) was tried. The rats, which weighed about 100 gm. at the beginning of the experiment, ceased to grow on these diets.

The concentration of ascorbic acid is expressed per gm. dry weight, as the water content of the livers of animals on the semi-synthetic diet receiving carcinogenic compounds, and that of the tu-

TABLE II: INJECTION EXPERIMENTS IN RATS, WITH 2-ACETAMIDOFLUORENE

Experiment	Day of injection	Killed on day after first injection	Ascorbic acid in liver, μgm. per gm. wet weight — Male: Control	A.A.F.	7-OH —A.A.F.	Female: Control	A.A.F.	7-OH —A.A.F.
			BLACK-AND-WHITE					
1	1,3	5	527	418	...	234	297	...
2	1,5	6	294	355	...	275	330	...
		8	296	319	...	244	200	...
3	1,4	5	268	398	385	223	364	240
		8	201	392	246	235	345	226
4	1,4	5	274	350	310	228	385	272
		8	226	319	290	228	318	284
		Mean	270	364	308	238	320	255
			100	135	114	100	134	107
			WISTAR					
1	1,4	5	176	266	...	249	178	...
		8	332	425	...	259	273	...
2	1,4	5	310	277	...	233	288	...
		8	362	195	...	240	295	...
3	1,4	5	204	320	276	179	259	205
			228	...	258	225	293	266
		Mean	269	297	267	232	263	235
			100	110	...	100	113	...

TABLE III: ASCORBIC ACID CONTENT OF LIVERS FROM WISTAR RATS ON SEMI-SYNTHETIC AND RICE DIETS

Compound added	Sex	Number of rats	Liver weight per cent of body weight Mean	Total solids per cent Mean	Ascorbic acid: μgm./gm. dry weight of liver Range	Mean	P.	μgm./gm. body weight Range	Mean	P.
SEMI SYNTHETIC DIET										
None	♀	6	3.5	32.1	675– 870	754	....	7.0– 9.2	7.8	....
	♂	4	3.8	32.4	695– 836	782	....	6.5– 9.2	8.3	....
7-OH-2Acet-amidofluorene.	♀	6	3.7	32.2	628– 995	796	0.5 –0.6	7.3–11.8	9.6	0.05–0.02
	♂	6	4.4	32.9	555–1005	801	0.8 –0.7	9.1–13.5	11.3	0.01
2-Acet-amidofluorene.	♀	7A	5.0	29.2	655–1441	885	0.02	10.3–16.9	13.3	<0.01
	♀	4B	6.5	29.8	675–1290	956	<0.01	13.3–19.8	17.1	....
	♀	2C	..	25.6	580–1190	885	....	....	...	....
	♂	5A	5.8	27.2	513–1320	998	<0.01	6.9–24.2	15.4	<0.01
	♂	4B	6.2	25.9	698–1270	890	0.1 –0.05	12.2–19.0	14.9	....
	♂	1C	..	27.5	....	455	....	....	..	....
p-Dimethylamino-azobenzene	♀	8B	4.3	27.4	565–1280	947	0.05–0.02	13.3–32.0	21.9	<0.01
	♀	6C	..	20.5	495– 910	697	....	....	..	....
	♂	2A	5.8	28.2	1118–1330	1224	<0.01	10.2–25.0	17.6	....
	♂	7B	5.0	27.4	730–1390	941	0.2 –0.1	11.4–22.7	18.1	0.01
	♂	6C	..	20.3	380–1340	940	....	....	..	....
RICE DIET										
p-Dimethylamino-azobenzene	♀	8	3.4	29.5	463– 930	649	....	4.3– 9.9	7.0	....
	♀	3B	5.2	27.8	710– 770	730	0.2 –0.3	10.8–10.9	10.1	0.02–0.01
Azobenzene	♀	7	4.4	31.0	840–1310	933	<0.01	8.5–16.7	11.8	<0.01
p-Dimethyl-aminoazo-benzene Rice + 6% casein	♀	7	3.5	30.6	677– 786	739	....	5.5–11.0	7.8	....
		8A	4.8	29.7	740–1010	864	0.05–0.1	10.4–22.7	14.3	<0.01
		12B	4.9	29.5	670–1170	863	0.05–0.1	9.4–16.1	11.9	<0.01
		1C	..	19.8	....	900	....	....	..	....

A—Livers without tumors. B—Normal parts of livers bearing tumors. C—Tumor tissue.

mors, was generally higher than that of the normal liver. In view of the increase in liver weight in the treated animals, the ascorbic acid content of the liver is expressed as μgm. per gm. of body weight, and not as the whole content of the liver.

RATS ON A SEMISYNTHETIC DIET. (TABLE III)

The water content of the liver was increased by *p*-DA and AAF, but not by 7-OH-AAF. In the group receiving AAF the ascorbic acid concentration (dry weight) is increased significantly in both sexes, as in those animals receiving *p*-DA, except in tumor-bearing males, where the result is perhaps affected by the presence of necrotic tissue.

When these data were calculated on a wet weight basis, no significant increase was found in either group. The figures are therefore omitted from the table.

Rats receiving 7-OH-AAF show no appreciable variation from the control group on either a dry or a wet weight basis.

The ascorbic acid content of the liver (μgm./gm. body weight) showed a striking increase among all animals receiving *p*-DA and AAF and a slight increase in those receiving 7-OH-AAF.

A concentration of ascorbic acid (dry weight) as high as that in the liver appeared in some of the tumors.

RATS ON RICE DIET. (TABLE III)

The concentration of ascorbic acid was greatly increased (wet and dry weight) by AB; was not increased by *p*-DA (wet weight) and only but barely so in some of the series on the dry weight basis.

The ascorbic acid content of the livers, calculated as μgm. per gm. of body weight, was raised significantly by AB, and by *p*-DA (up to 100 per cent in one series). The water content of the liver was not altered.

DISCUSSION

Differences in results corresponding to differences in species, strain, sex and method of administration make generalization difficult in this subject as in other branches of cancer research. The data now available are summarized in Table IV. In earlier short-term (2 to 19 days) experiments (8) injection of seven carcinogens increased the concentration of ascorbic acid in mice; the very slightly carcinogenic 1,2-benzanthracene was equally active, while the three non-carcinogenic compounds (anthracene, phenanthrene, naphthalene) gave negative results. The investigation has now been extended to include short-term injection and long-term feeding experiments on rats. No short-term injection experiment comparable to those described

above was made with AB, which so far as we know is non-carcinogenic, but the ascorbic acid was increased by long-term feeding with this compound. *p*-DA gave a more definite positive result when fed to rats than when injected in mice (8). A very distinct change is produced in AAF by the insertion of the 7-OH group, giving a compound which is much less carcinogenic (Hoch-Ligeti, unpublished experiments) and in which the power to increase the ascorbic acid in the liver is much diminished. The greater response to the injection of AAF in black-and-white in contrast to Wistar rats (Table II) is perhaps of interest in view of the increased susceptibility of the latter to the induction of mammary tumors (1).

TABLE IV: SUMMARY OF RESULTS

Compound	Method of administration	Species and strain	Ascorbic acid in liver*
3,4-Benzpyrene	Injection subcut. and intraperit.	CBA ♂ mice	+
Cholanthrene	Injection intraperit.	" " MRC ♀ "	+
Methyl cholanthrene	" "	" " CBA ♀ "	+
9,10-Dimethyl-1:2- Benzanthracene	" "	CBA ♀ "	+
1,2,5,6-Dibenzanthracene	" "	MRC ♂ "	+
1,2,5,6-Dibenzphenanthrene	" "	" " "	+
Stilbestrol	" "	" " "	+
1,2-Benzanthracene	" "	CBA ♂ "	+
2-Acetamidofluorene	" subcut. and intraperit.	" " "	+
"	" " "	" ♀ "	−
"	" intraperit.	Blace & White rats ♂ and ♀	+
"	" "	Wistar rats ♂ and ♀	+(slight)
"	Fed.	" " " " "	+
p-Dimethylaminoazobenzene	Injection intraperit.	CBA ♂ and ♀ mice	±
"	Fed.	Wistar rats ♂ and ♀	+
2,2′-Azonaphthalene	Injection intraperit.	Stock ♂ mice	+
Azobenzene	Fed.	Wistar ♀ rats	+
9,10-Dimethylanthracene	Injection intraperit.	MRC ♀ mice	−
Anthracene	" "	CBA ♀ "	−
Phenanthrene	" "	MRC ♂ "	−
Naphthalene	" "	" " "	−
7-OH-Acetamidofluorene	" "	CBA ♂ and ♀	−
"	" "	Black & White rats ♂ and ♀	+(very slight)
"	" "	Wistar rats ♂ and ♀	−
"	Fed.	" " " " "	−

* + indicates an increase, − no increase, ± an inconclusive result.

From the values for ascorbic acid, and for water content of the liver (Table III), it would appear that in animals on a semi-synthetic diet, in which hepatic tumor development is rapid, a definite increase in ascorbic acid is accompanied by a rise in the water content of the liver tissue, whereas animals on a rice diet that develop tumors slowly show a barely significant rise in ascorbic acid and no change in water content. The result of the short-term experiments (Tables I and II) indicate that the increase in ascorbic acid precedes any rise in water content. Hence it would seem that increases in ascorbic acid and water content of the liver are related to the rate of tumor growth, although it is possible that all three effects are due to nutritional causes.

The level of ascorbic acid following administration of carcinogenic substances rose sharply after injection in black-and-white rats (Table II), and after feeding in Wistar rats, and reached a value at which it remained raised. As the ascorbic acid content of the cells in embryonic tissue is increased during active histogenesis (ref. 2, page 136—$AgNO_3$ method) changes in the liver produced by carcinogenic compounds may resemble those of differentiation from one tissue into another. Support for the supposition that changes in the differentiation process of liver cells occur soon after feeding carcinogens might be drawn from the fact that the withdrawal of *p*-DA from the diet after about 50 days does not arrest the development of tumors.

An alternative explanation for the increase in ascorbic acid might be drawn from consideration of cell metabolism. During increased metabolic processes ascorbic acid accumulates in the Golgi apparatus of the cell according to Bourne (2, page 36). The rise in the ascorbic acid content of the liver tissue on administration of carcinogens might be, therefore, an expression of increased metabolic activity, possibly connected with detoxication of the carcinogen. This suggestion might account also for the increase in ascorbic acid content observed on feeding azobenzene. This compound is not car-

cinogenic but nevertheless may provoke similar detoxification processes.

SUMMARY

The effect of carcinogens on the ascorbic acid content of the liver has been further investigated. 2-Acetamidofluorene increased the ascorbic acid content of the liver in CBA male mice, in black-and-white rats, and to a less extent in Wistar rats, when injected, and in Wistar rats when fed; 7-OH-2-acetamidofluorene was much less active.

The ascorbic acid content was increased when *p*-dimethylaminoazobenzene and azobenzene were fed to Wistar rats.

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