The Binding of *o*-Aminoazotoluene to Deoxyribonucleic Acid, Ribonucleic Acid and Protein in the C57 Mouse

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1. ^3H -labelled *o*-aminoazotoluene was synthesized from $[\text{G-}^3\text{H}]\text{o-toluidine}$ on a semi-micro scale. 2. An association of ^3H with DNA, RNA and protein from the liver, kidney and spleen of female C57b mice was demonstrated after the administration of a single dose of $[\text{G-}^3\text{H}]\text{o-aminoazotoluene}$. 3. This association is judged to represent covalent binding as a result of experiments involving solvent extraction, examination of the acid hydrolysates of the DNA and RNA and administration of $[\text{G-}^3\text{H}]\text{o-aminoazotoluene}$. 4. Examination of the extents of binding at various times after the administration of a single dose of $[\text{G-}^3\text{H}]\text{o-aminoazotoluene}$ showed that there was a peak of binding to liver DNA in the female mice at about 16 hr. that was not present in the male mice. 5. The extent of binding to DNA, RNA and protein at 16 hr. in the female C57b mouse liver was greater than that in the spleen and kidney.

The covalent binding of many chemical carcinogens to cellular macromolecules has been described (Brookes, 1966), but has almost exclusively been restricted to two situations, namely the binding of hepatocarcinogens in rat liver (Miller & Miller, 1953; Magee & Farber, 1962; Roberts & Warwick, 1966; Warwick, 1967) and of cutaneous carcinogens in mouse skin (Brookes & Lawley, 1964; Heidelberger, 1964). The present study was begun to widen the knowledge of this type of reaction and to ascertain whether binding in the liver, which is well established in the rat, could be similarly established in the mouse.

o-AAT* (I) is a liver carcinogen in mice (Andervont & Dunn, 1947), rats (Yoshida, 1932), dogs (Nelson & Woodard, 1953), Syrian hamsters (Tomatis, Della Porta & Shubik, 1961) and rainbow trout (J. E. Halver, personal communication). It has been reported that *o*-AAT binds covalently with rat liver proteins (Terayama, Teruya, Kusama

& Aoki, 1958). There is a well-defined difference in the susceptibility of the sexes in the C57 mouse to the carcinogenic activity of *o*-AAT, females being more prone to the induction of liver tumours than males. This provides a useful situation in which to examine the correlation between carcinogenicity and other reactions induced by *o*-AAT.

MATERIALS AND METHODS

Radioactive chemicals. $[\text{G-}^3\text{H}]\text{Thymidine}$ (5 c/m-mole) and $[\text{G-}^3\text{H}]\text{o-toluidine}$ were obtained from The Radiochemical Centre, Amersham, Bucks. Two batches of *o*-toluidine were received, the first as *o*-toluidine sulphate (1 mc/mg.) and the second as *o*-toluidine hydrochloride (20 mc/mg.). *o*-Toluidine sulphate was converted into *o*-toluidine by double decomposition with NaOH and extraction of the liberated *o*-toluidine into ether. *o*-Toluidine hydrochloride was used as such. In the synthesis of *o*-AAT, $[\text{G-}^3\text{H}]\text{o-toluidine}$ or its hydrochloride was diluted threefold with non-radioactive *o*-toluidine or its hydrochloride.

Preparation of $[\text{G-}^3\text{H}]\text{o-AAT}$. *o*-Toluidine (200 mg.) or the hydrochloride (266 mg.) was thoroughly mixed with 11 N-HCl (0.2 ml.) by mechanical agitation and cooled in an ice bath. Cooled NaNO_2 (65 mg. in 0.2 ml. of water) was added dropwise and the mixture was agitated. The temperature was maintained below 5°. When all the NaNO_2 had been added, the reaction was stopped by the addition of ether (5 ml.). The ether extract was streaked on to thin-layer films of silica (Kieselgel PF₂₅₄; E. Merck A.-G., Darmstadt, Germany) of 1.5 mm. thickness and developed in benzene. When sufficient separation had been achieved, the band containing the *o*-AAT was scraped

(I) *o*-Aminoazotoluene

*Abbreviation: *o*-AAT, *o*-aminoazotoluene.

from the still damp plate and the *o*-AAT was eluted from the silica with methanol, which was removed by evaporation. The crude preparation was dissolved in benzene and stored until required, when it was purified by further thin-layer chromatography.

When *o*-toluidine of higher specific radioactivity (20 mc/mg.) was used, the ether extract of the reaction mixture was purified on an alumina column (20 cm. $\times$ 2.5 cm.), portions of the benzene eluate being further purified by thin-layer chromatography immediately before use.

The yield of *o*-AAT was 35% and the recovery of radioactivity was 25%. The specific radioactivity in the first preparation was 12.8 mc/m-mole and that in the second preparation was 110 mc/m-mole. The latter was diluted to 12.8 mc/m-mole before use.

A sample of unlabelled *o*-AAT prepared by this method was an orange-red powder (m.p. 99–100°). Microanalysis, performed by Mr J. Stewart, Department of Organic Chemistry, University of Leeds, gave: C, 75.1; H, 6.5; N, 18.6. Calc. for $C_{14}H_{15}N_3$: C, 74.7; H, 6.7; N, 18.6%. The u.v. and visible spectra of this material and the 3H -labelled material were identical with those of purified commercial *o*-AAT.

In an attempt to obtain the maximum yield of *o*-AAT, the experimental conditions were varied. Increasing the pH decreased the yield; increasing the concentration of $NaNO_2$ above the calculated amount (in an attempt to utilize any unchanged *o*-toluidine) also caused a decrease in the yield. The length of time for which the reaction was allowed to proceed was varied, and the optimum time was 5 hr.

Animals. Male and female C57 mice, bred in this Department, were maintained on Oxoid 41B diet and water *ad libitum*. They were 10–12 weeks old when used.

Conduct of animal experiments. [$G-^3H$]Thymidine was administered by intraperitoneal injection (0.2 μ C/g.; 5 c/m-mole). [3H]*o*-AAT was administered by gastric incubation in arachis oil (0.4 μ C, 7 μ g./g.). The animals were starved for 16 hr. before death to lower the amount of glycogen in the liver, and were killed by prolonged ether anaesthesia. The tissues were rapidly dissected and immediately frozen by immersion in liquid N_2 .

Preparation of nucleic acids and protein fractions. Three preparation methods were used. Two were used to prepare DNA, RNA and protein for the examination of binding, and gave similar results. The third method was used to prepare DNA for the examination of the incorporation of [3H]thymidine.

Preparation 1. Nucleic acids (RNA and DNA) were prepared by the modification of the method of Kirby (1962) described by Craddock, Villa-Trevino & Magee (1968). Protein was prepared by a modification (Magee & Farber, 1962) of the Schneider (1945) procedure.

Preparation 2. The frozen tissue was homogenized in tri-isopropyl-naphthalenesulphonic acid (sodium salt). RNA and DNA were prepared by the method of Parrish & Kirby (1967), the treatment with naphthalene-1,5-disulphonic acid (sodium salt), pH 6.5, being omitted. Protein was isolated as in preparation 1.

The DNA contained not more than 2% of protein and the RNA not more than 1% of protein; the protein was free of nucleic acids.

Preparation 3. DNA was prepared for the examination

of thymidine incorporation by the method of Schmidt & Thannhauser (1945).

Spectrophotometric determinations. DNA was estimated by the diphenylamine method of Burton (1956). Spectrophotometric determinations were performed on a Unicam SP. 500 spectrophotometer.

Radioactivity measurements. Radioactivity measurements were made in a three-channel liquid-scintillation spectrometer with automatic external standardization (model 3314; Packard Instrument Co., Downers Grove, Ill., U.S.A.), by using a scintillation fluid consisting of 2,5-diphenyl-oxazole (5.0 g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.3 g.) in 1 l. of redistilled toluene. The samples were prepared for counting by solubilization in Hyamine hydroxide 10 X [Nuclear Enterprises (G.B.) Ltd., Edinburgh].

Hydrolysis of nucleic acids. Nucleic acids were hydrolysed in 1 N-HCl at 100° for 1 hr. (Vischer & Chargaff, 1948).

Measurement of radioactivity on paper chromatograms. Because of the low specific radioactivity of the [3H]*o*-AAT, the hydrolysates of RNA and DNA, containing bound radioactivity, were applied to papers as streaks to obtain as high an amount of material as was consistent with resolution. The chromatograms were developed in butan-1-ol-acetic acid-water (12:3:5, by vol.). When development was complete, the papers were dried and divided into strips 1 cm. wide, across the width of the paper. These strips (20 cm. $\times$ 1 cm.) were rolled into coils and placed flat in glass vials, containing 10 ml. of scintillation fluid for counting in the liquid-scintillation counter. The coils were always laid in the same way to prevent any errors due to different orientation in the vials (Davidson, 1962).

Extraction of nucleic acids and protein with organic solvents. Samples of RNA, DNA and protein, in 2.5 M- K_2HPO_4 buffer, pH 7.4, were extracted with ether and benzene in a liquid-liquid extractor. Unbuffered solutions were degraded by solvolysis when extracted with ether.

RESULTS

All the values referring to binding experiments are the means of at least three experiments, each one involving a group of eight to ten mice.

The binding of [3H]*o*-AAT to DNA in female C57 mice rose to a maximum about 16 hr. after dosing (Table 1). The decline was rapid at first and then more gradual. The binding to RNA declined throughout the experiment, whereas the binding to protein followed a curve that was difficult to interpret. Extraction of the samples of bound RNA, DNA and protein with ether for 5 hr. removed 12% of the radioactivity from the DNA, 14% from the RNA and 8% from the protein, and similar extraction with benzene removed 12% of the radioactivity from the RNA and DNA and 8% from the protein, indicating that only small amounts of the radioactivity associated with the isolated constituents were due to absorption of the dye or its metabolites.

Hydrolysis of the DNA and RNA followed by paper chromatography showed that the radio-

Table 1. Amounts of radioactivity in liver DNA, RNA and protein of female C57 mice after oral administration of [^3H]o-AAT (0.4 $\mu\text{C/g.}$)

Time after [^3H]o-AAT (hr.)	No. of expts.	Radioactivity (mean $\pm$ s.d.) (nc/c.)		
		DNA	RNA	Protein
6	3	13 $\pm$ 3	72 $\pm$ 25	84 $\pm$ 7
12	3	24 $\pm$ 4	59 $\pm$ 11	255 $\pm$ 28
16	4	77 $\pm$ 9	27 $\pm$ 4	83 $\pm$ 3
20	3	36 $\pm$ 10	21 $\pm$ 5	85 $\pm$ 6
32	3	24 $\pm$ 5	28 $\pm$ 5	120 $\pm$ 3
64	3	18 $\pm$ 6	17 $\pm$ 6	30 $\pm$ 3

activity was separable from the natural bases and nucleotides, indicating that the radioactivity was initially covalently bound and had not been incorporated into the major normal components of the nucleic acids after degradation of the dye. Further evidence that the radioactivity was not present by incorporation of ^3H was obtained when female C57 mice were given unlabelled o-AAT (7 $\mu\text{g./g.}$) and [^3H]water (0.4 $\mu\text{C/g.}$) by gastric intubation. The amounts of radioactivity associated with the RNA, DNA and protein from the livers of these mice after 16hr. were 1.5nc/g. of DNA, 1.3nc/g. of RNA and 10.5nc/g. of protein. These values were 2%, 4% and 13% respectively of those obtained after administration of [^3H]o-AAT.

To eliminate the possibility that the binding resulted from the method of preparation of the samples, the livers from a group of female C57 mice that had been starved for the previous 16hr. were homogenized with [^3H]o-AAT (6 μC), i.e. one-tenth of the dose that this group of mice would have been given. The nucleic acids and protein were prepared, but no radioactivity was associated with any of the macromolecular constituents.

The failure of o-AAT to produce tumours of the spleen and the kidney in female C57 mice (Andervont & Dunn, 1947) can be used to examine a correlation between the binding and carcinogenicity. Table 2 shows that the binding to DNA, RNA and protein was much less in the spleen and the kidney than in the liver.

o-AAT is more carcinogenic in female mice than male C57 mice (Andervont, Grady & Edwards, 1942), which makes available another system in which to examine a possible correlation between carcinogenicity and binding. The binding to liver DNA, RNA and protein (Table 3) shows that in male C57 mice, the binding to DNA rose to a maximum about 16hr. after dosing, but this value was much lower than the binding in the female at this time (Table 1). Further, the binding in the male mice did not decline from the value at 16hr., but

Table 2. Amounts of radioactivity in DNA, RNA and protein from liver, kidney and spleen of female C57 mice 16hr. after the oral administration of [^3H]o-AAT (0.4 $\mu\text{C/g.}$)

Tissue	No. of expts.	Radioactivity (mean $\pm$ s.d.) (nc/g.)		
		DNA	RNA	Protein
Liver	4	77 $\pm$ 9	27 $\pm$ 4	83 $\pm$ 3
Kidney	3	29 $\pm$ 12	16 $\pm$ 2	36 $\pm$ 9
Spleen	3	12 $\pm$ 3	11 $\pm$ 4	16 $\pm$ 4

Table 3. Amounts of radioactivity in liver DNA, RNA and protein of male C57 mice after oral administration of [^3H]o-AAT (0.4 $\mu\text{C/g.}$)

Time after [^3H]o-AAT (hr.)	No. of expts.	Radioactivity (mean $\pm$ s.d.) (nc/g.)		
		DNA	RNA	Protein
12	3	4 $\pm$ 2	15 $\pm$ 7	28 $\pm$ 5
16	3	24 $\pm$ 10	26 $\pm$ 8	85 $\pm$ 24
20	3	13 $\pm$ 7	21 $\pm$ 10	61 $\pm$ 17
32	3	22 $\pm$ 8	28 $\pm$ 5	68 $\pm$ 21

Table 4. Incorporation of [^3H]thymidine into liver DNA of male and female C57 mice previously fed with unlabelled o-AAT (7 $\mu\text{g./g.}$)

[^3H]Thymidine was given 1hr. before death.

Time after o-AAT (hr.)	No. of mice	Radioactivity (mean $\pm$ s.d.) (disintegrations/min./ $\mu\text{g.}$ of deoxyribose)	
		Male	Female
0	16	30 $\pm$ 7	20 $\pm$ 4
12	16	28 $\pm$ 7	23 $\pm$ 4
16	16	27 $\pm$ 6	34 $\pm$ 16
20	16	27 $\pm$ 7	24 $\pm$ 9

remained approximately constant until 32hr. after the administration of the dye, when the experiment was terminated. The binding to RNA also rose to a maximum after 16hr. and remained constant, in contrast with the binding to RNA in female mice. The binding to protein did not follow the undulating curve of the binding to protein in female mice.

The difference in binding to DNA in the male and female mice over the first 32hr. did not appear to be due to differences in DNA replication. The incorporation of [^3H]thymidine into liver DNA of female and male mice that had been fed with

unlabelled *o*-AAT (7 μ g./g.) (Table 4) showed a small peak of DNA synthesis in the female during this period, which was statistically significant ($0.01 > P > 0.001$ by the *t* test), but was probably insufficient to account for the sharp peak in the binding of *o*-AAT to DNA in the female.

DISCUSSION

It has been shown that radioactivity is associated with the nucleic acids and protein of C57 mouse liver after the administration of a single dose of [3 H]*o*-AAT. The radioactivity probably represents covalently bound *o*-AAT or a metabolite, as other explanations were experimentally excluded. The absorption of the dye or its metabolite on to the macromolecule is ruled out by the failure to remove appreciable amounts by extraction with ether or benzene. The possibility that the radioactivity associated with the nucleic acids was due to incorporation of 3 H that had been released by degradation of the dye is unlikely, because the radioactivity was not associated with the bases and nucleotides formed by acid hydrolysis of the nucleic acids. Also, very little radioactivity was incorporated into the macromolecules after the administration of unlabelled *o*-AAT and [3 H]water. The radioactivity associated with the macromolecules was not an artifact of the method of isolation, as no radioactivity was associated with the nucleic acids and protein after they had been isolated from the livers of untreated mice in the presence of [3 H]*o*-AAT. Direct proof of covalent binding will depend on the isolation and characterization of the adducts of *o*-AAT and the macromolecules.

The binding to DNA after the administration of a single dose of [3 H]*o*-AAT showed in the female C57 mouse a marked peak between 12 and 20 hr. that was absent in the male. This difference in the sex response to the dye is probably of a more fundamental nature than a mere difference in metabolism of the dye, because the values of binding to DNA were approximately the same in the male and female once the peak of binding in the female had passed. The possibility that the fall in binding in female mice between 16 and 20 hr. was due to an increased rate of DNA synthesis is unlikely. Although there was, in the female, a slight increase in [3 H]thymidine incorporation into DNA at 16 hr. this was insufficient to account for the fall in specific radioactivity of the DNA from animals treated with [3 H]*o*-AAT, unless the binding

of the dye or its metabolite occurs with a small part of the total DNA. Similar peaks of binding of dyes to DNA of rat liver have been observed previously (Roberts & Warwick, 1966; Warwick, 1967). These varied in their time of appearance, tending to occur later than the peak of binding of *o*-AAT, and also in their magnitude. There does not appear to be a previous report of a difference between the sexes in the formation of bound dye, although 4-dimethylaminoazobenzene, one of the dyes used in similar studies (Roberts & Warwick, 1966), is a more potent liver carcinogen in male rats than in female rats (Mulay & O'Gara, 1959).

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