Metabolism of Bladder Carcinogens

3. THE METABOLIC PATH OF 2-[8-¹⁴C]NAPHTHYLAMINE IN SEVERAL ANIMAL SPECIES*

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(Received 27 April 1960)

In previous papers (Henson, Somerville, Farquharson & Goldblatt, 1954; Somerville, Henson, Cooke, Farquharson & Goldblatt, 1956) we reported the results of investigations into the distribution of 2-[8-¹⁴C]naphthylamine in the rat and rabbit, after intraperitoneal injection. We now present the results of similar studies on several different animal species, together with some observations on the absorption of the material into the blood stream through the skin and through the bladder wall.

MATERIALS AND METHODS

These were, with slight modification, as previously described.

Preparation of 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate. This was prepared by feeding 2-[8-¹⁴C]naphthylamine (+ inactive naphthylamine) to a dog and extracting the sulphuric ester from the urine. A portion (50 ml.) of the urine, adjusted to pH 4 with acetic acid, was passed through a column of charcoal deactivated with 4% stearic acid and the charcoal was washed with water. The adsorbed material was eluted with 100 ml. of saturated aq. phenol and 100 ml. of water and evaporated to dryness. It was repeatedly dissolved in water and evaporated to dryness and finally dried for several days *in vacuo* to remove the phenol. The brown, gummy residue was dissolved in butanol-ethanol-water (17:3:20, by vol.), made into a slurry with cellulose powder, and the slurry was placed on top of a dry cellulose powder column and eluted with the same solvent. The 2-amino-1-naphthyl hydrogen sulphate moves close behind the solvent front, so the first eluate fraction was removed and evaporated. The residue was boiled with ethanol, in which it was largely insoluble. Filtration and drying left about 90 mg. of a brown, powdery solid, which was shown by paper chromatography to consist mainly of the sulphuric ester; much smaller amounts of several slower-running materials were present but these were non-radioactive. The activity of the 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate [assayed as described by Henson *et al.* (1954) and Somerville *et al.* (1956)] was 4.87×10^4 counts/min./mg.

Oxidation techniques. We have found exposure to hot reduced copper to be an unreliable method of removing

oxides of nitrogen from CO₂ prepared from animal tissues for measurement in Geiger counters. Absorption by manganese dioxide (Belcher & Ingram, 1950; Christman, Day, Hansell & Anderson, 1955) has been found to give much more consistent results and to be a simpler method to apply.

RESULTS

Excretion of radioactivity from several animal species after intraperitoneal injections of 2-[8-¹⁴C]naphthylamine. The percentages of the dose excreted in the urine and faeces of dogs, guinea pigs and mice are shown in Table 1. Figures are given for excretion after 2 days (approx.) and for total excretion (after daily amounts had become negligible).

Table 1. *Percentage of dose excreted in urine and faeces of dogs, guinea pigs, mice, rabbits and rats after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Doses: about 0.1 mg. to mice, 0.3–1.0 mg. to guinea pigs, rats and rabbits and 6 mg. to dogs.

Species	Percentage of dose excreted			
	Urine		Faeces	
	0–2 days	Total	0–2 days	Total
Dog	85.6	87.4	1.4	2.5
	74.0	75.4	3.0	3.2
Guinea pig	64.4	65.8	10.9	12.8
	78.4	79.9	11.0	14.2
	86.9	88.0	5.8	7.9
	79.2	80.0	5.4	6.4
Mouse	81.0	82.2	9.1	10.1
	77.2	78.4	10.7	12.8
	70.7	72.2	16.8	16.9
Rabbit*	81.7	—	7.8	—
	88.4	89.9	2.6	7.4
	81.8	82.4	12.5	13.3
Rat†	65.5	67.0	22.2	28.4
	60.9	61.8	26.5	28.1

* Part 2. Somerville, Henson, Cooke, Farquharson & Goldblatt (1956).

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* Values from Somerville, Henson, Cooke, Farquharson & Goldblatt (1956).

† Values from Henson, Somerville, Farquharson & Goldblatt (1954).

Chromatography of urine. Urines were collected for 24 hr. from a dog, rabbit, guinea pig, rat and mouse which had received intraperitoneal injection of 2-[8- 14 C]naphthylamine, and were applied to Whatman 3MM paper, the volume for each animal being such that all spots contained about the same amount of radioactivity. One-dimensional chromatograms were run with two-solvent systems: butan-1-ol saturated with 2N-NH₃ soln., and butan-1-ol-ethanol-water (17:3:20, by vol.). The radioautographs of the chromatograms of the dog, rabbit, guinea pig, rat and mouse urines showed 1, 4, 6, 7 and 7 spots respectively and the most intense spots from dog, rat and mouse urine had the same R_f as did 2-amino-1-naphthyl sulphate.

Activity in blood and bile of a dog and a cat after

Fig. 1. Concentration of activity in blood and bile of dog 1 after intraperitoneal injection of 2-[8- 14 C]naphthylamine. O, Blood; ●, bile. Dose: 3.20×10^7 counts/min.

intraperitoneal injection of 2-[8- 14 C]naphthylamine. The bile ducts of a dog and of a cat were cannulated and bile was collected continuously after intraperitoneal injection of naphthylamine. Blood samples were taken periodically. The variation with time of the activity in blood and in bile are shown in Figs. 1 and 2; the patterns are similar to that for rabbits (Somerville *et al.* 1956, Fig. 3). The ratio of the concentration of activity in bile to that in blood, in the period after the peak level has been passed, varies from about 30 to 60 for the dog and 100 to 200 for the cat. Corresponding figures for the rabbit were 3-30 (Somerville *et al.* 1956) and for a rat 40-70. The total activity excreted in the bile of the dog

Fig. 2. Concentration of activity in blood and bile of cat 3 after intraperitoneal injection of 2-[8- 14 C]naphthylamine. O, Blood; ●, bile. Dose: 3.39×10^7 counts/min.

Table 2. *Distribution of activity between blood plasma and cells in guinea pigs after intraperitoneal injection of 2-[8- 14 C]naphthylamine*

Results are expressed as counts/min./mg. dry wt. Dose to each guinea pig: 4.21×10^7 counts/min. (about 1.5 mg.). Animals weighed 600-700 g.

Time after injection (days)	Guinea pig 9		Guinea pig 10		Guinea pig 11		Guinea pig 12	
	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells
2	1.4	—	1.4	2.2	1.3	1.7	1.7	1.3
6	0	2.0	0.6	1.4	—	1.4	0.3	1.2
9	—	1.7	1.1	0.9	0.6	1.0	0	0.8
13	0	2.2	0	1.2	0	0.8	—	1.0
16	0.4	1.4	0	1.2	0	1.0	0	0.8
20	0.4	1.4	0.2	1.1	0.2	1.1	0.4	1.2
23	0	—	0	0.8	0	0.8	0	0.7
27	—	1.3	—	0.8	—	0.6	—	1.0
30	—	1.6	—	0.9	—	0.7	—	0.4
34	—	1.2	—	0.8	—	0.8	—	1.1
37	—	0.8	—	1.3	—	1.3	—	1.0
41	—	1.0	—	0.5	—	0.3	—	0.4
44	—	0.9	—	0.7	—	0.4	—	0.2
48	—	0.6	—	0.5	—	0.5	—	0.3
51	—	0.5	—	0.3	—	0.3	—	0.4
54	—	0.5	—	0.3	—	0.2	—	0.2
61	—	0.4	—	0	—	0.1	—	0.1
68	—	0.2	—	0.1	—	0	—	0.2

Table 3. *Distribution of activity between blood plasma and cells of a beagle dog after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Wt. of dog, 10 kg. Dose: 2.16×10^8 counts/min. (about 8 mg.). Results are expressed as counts/min./mg. dry wt. (Note: counting rates for samples from plasma after 22 days were only slightly above the minimum detectable level; accuracy of these results is therefore low.)

Time after injection (days)	Plasma	Cells
1	42.0	4.3
2	19.0	2.6
3	7.4	2.8
4	—	7.1
7	2.0	1.2
8	1.7	0.9
9	1.3	0.7
11	1.2	—
15	—	0.7
22	0.5	1.1
27	0.2	1.1
36	0.4	0.6
43	0.3	0.6
52	0.8	0.4

Table 4. *Distribution of activity between plasma, cell contents and cell membranes of rat blood after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Blood of rat 45 was taken 13 days and of rat 46 20 days after injection. Dose to each rat: 1.82×10^7 counts/min. (about 0.7 mg.). Results are expressed as counts/min. in the amount of blood constituent contained in 1 g. of whole blood.

	Rat 45	Rat 46
Plasma	37	22
Cell contents	3932	2900
Cell membranes	12	8

Table 5. *Activity in the blood of rat 33 after application of 2-[8-¹⁴C]naphthylamine to the skin*

Dose: 3.59×10^7 counts/min. (about 1.3 mg.). Results are expressed as counts/min./mg. wet wt.

Time (days)	...	1	2	3	6	7	8	9
Activity in blood		2.6	4.9	3.8	0.3	1.5	3.4	2.8
Time (days)	...	10	13	14	17	21	24	28
Activity in blood		2.9	2.1	2.0	1.7	1.4	1.1	0.7

in 3 hr. was 1.2 % of the dose and 2.2 % was found in the bile in the gall bladder. Corresponding figures for the cat are 12.2 and 0.03 % respectively in 2½ hr.

Partition of activity between blood constituents of dogs, guinea pigs and rats. Somerville *et al.* (1956) have shown that in rat blood the activity remains firmly attached to the cells after it has disappeared from the plasma, whereas in rabbits the activity disappears from the cells at about the same time as

Fig. 3. *Concentration of activity in blood of rats after application of 2-[8-¹⁴C]naphthylamine to skin. ●, Rat 32; dose: 4.54×10^7 counts/min. ○, Rat 33; dose: 3.60×10^7 counts/min.*

from the plasma. Results for guinea pigs and a dog are given in Tables 2 and 3. The site of the radioactive material which is firmly bound to rat-blood cells was further investigated by separating the cell contents from the membranes. Blood was taken from two rats at 13 and 20 days respectively after the intraperitoneal injection of naphthylamine. The cells were separated by centrifuging, washed twice with sodium citrate solution (22 g./l.) and then haemolysed with 6.7 mN-NH₃ soln. Cell contents and membranes were separated by centrifuging and the membranes washed five times with the NH₃ soln., the washings being added to the cell contents. Activity in the plasma, cell contents and cell membranes was measured (Table 4).

Activity in blood of rats and of a dog after application of 2-[8-¹⁴C]naphthylamine to the skin. Absorption of naphthylamine through the skin has been studied in the rat. The animal's back was shaved and radioactive naphthylamine dissolved in a small volume of ethanol was applied. After the solvent had evaporated the area was covered with adhesive tape and the animal held in a restriction cage for the first few hours. Blood samples were taken from the tail and in every case appreciable amounts of activity were detectable in the blood after 10 min. The variation in the activity in the blood of rats 32 and 33 in the first few hours after application is shown in Fig. 3. In one case the activity in the blood was followed over a longer period; the results are given in Table 5. The fraction of the dose excreted in the urine of rat 32 was 10.8 % in 5 hr., and in rat 33 it was 14.8 % in 3 days.

A similar application was made to the skin of a dog (wt. 10 kg.). With a dose of about 6×10^7 counts/min. (2 mg.) definite evidence was obtained of penetration through the skin, although the counting rates of the samples were only just above the level of significance (3 counts/min.). Thus after

1 hr. a blood sample gave 5 counts/min. (0.1 count/min./mg. wet wt.) and the highest counting rate recorded was 12 counts/min. (0.08 count/min./mg. wet wt.) after 30 hr. (The highest concentration in the blood occurred a few hours after the skin application, but smaller samples were taken.) Amounts of this order were still detectable after 7 days.

Absorption of 2-[8-¹⁴C]naphthylamine and of 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate from the bladder into the blood of cats. 2-[8-¹⁴C]Naphthylamine (2.05 mg.) was suspended in approx. 0.05M-KH₂PO₄ solution, dissolved with the minimum amount of HCl and the solution was brought to pH 3-4 with Na₂HPO₄. The total volume of 3 ml. was introduced by syringe and catheter into the bladder of an anaesthetized male cat, followed by 3 ml. of KH₂PO₄ solution, the bladder having previously been emptied of urine as thoroughly as possible after the introduction of the catheter. Blood samples were taken at intervals from the femoral vein. Concentrations of active material in the blood are shown in Table 6. If it is assumed that the total weight of blood was 5 % of that of the whole animal, the maximum concentration at 165 min. represents a fraction of about 3.3 % of the dose in the blood.

A similar experiment was performed with 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate, 54 mg. of which was dissolved in 4 ml. of KH₂PO₄ and Na₂HPO₄ solution (each approx. 0.05M) adjusted to pH 7 with Na₂CO₃ and introduced by catheter into the bladder of an anaesthetized male cat, followed by 3 ml. of sodium chloride solution (8.5 g./l.). Blood samples were taken at intervals from the cannulated jugular vein, and concentrations of activity in the blood are shown in Table 7. On the

same assumption as was made for cat 4, the maximum concentration at 220 min. represents a fraction of about 0.5 % of the dose in the blood.

DISCUSSION

The excretion of naphthylamine or its metabolites, after intraperitoneal injection, takes a similar course in dogs, guinea pigs, mice, rabbits and rats in that almost the whole dose is excreted in 2 days (Table 1), but small amounts remain in the body and are detectable in the blood for several weeks afterwards [Tables 2 and 3 and Somerville *et al.* (1956), Tables 1 and 2]. Since this long-term retention of a small fraction of the dose has been shown to occur in four animal species it probably also occurs in man, and may have been significant in the induction of tumours in workers exposed to 2-naphthylamine, for their bodies would never have been entirely free of the material. The distribution of the excreted radioactivity between faeces and urine varies between species. The dog excretes only about 3 % of the dose in the faeces, the guinea pig, mouse and rabbit about 10 % and the rat almost 30 %. The small amount of activity in the bile of the dog with cannulated bile duct is concordant with the low excretion in the faeces. The fact that the dog excretes the highest fraction of the dose in the urine may be significant, in that the dog is the only experimental animal in which bladder tumours have been induced by feeding naphthylamine, but it cannot be of major importance, for even the rat excretes about two-thirds of the dose in the urine.

The experiments recorded in Tables 6 and 7 show that naphthylamine was absorbed from the bladder of a cat to an extent about seven times that of 2-amino-1-naphthyl hydrogen sulphate. Since the 'true' carcinogen (whether amine or metabolite), in order to exert its action on the proliferating layers of the bladder epithelium, must penetrate the mucous membrane at least as far as the submucous layer, it is evident that the amine can attain the site of carcinogenic action much more readily than can the sulphate. We suggest that it is possible that the carcinogenic action in man and dog might be due to small amounts of unchanged amine excreted in the urine, for Bonser, Bradshaw, Clayson & Jull (1956) have shown by the bladder-implantation technique that it is a carcinogen, if a weak one, and D. Manson (personal communication) has detected significant amounts of the base in the urine of dogs fed 0.2 g. of naphthylamine. In any case the ability to penetrate to the proliferating layers of cells is a prime requirement for any potential carcinogen and other metabolites which have been suggested as the 'true' carcinogen, e.g. 2-amino-1-naphthol, 2-amino-1-naphthyl glucosiduronic acid and bis-(2-

Table 6. *Activity in the blood of cat 4 after introduction of 2-[8-¹⁴C]naphthylamine into the bladder*

Dose: 5.58×10^7 counts/min. (about 2 mg.). Wt. of cat, 2.5 kg. Results are expressed as counts/min./mg. wet wt.

Time after introduction (min.)	40	135	165	200	250
Activity in blood	8.0	14.0	14.5	12.8	9.0

Table 7. *Activity in the blood of cat 5 after introduction of 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate into the bladder*

Dose: 2.63×10^7 counts/min. (54 mg.). Wt. of cat, 2.0 kg. Results are expressed as counts/min./mg. wet wt. (Counting rates were low. Each value is the mean from two samples each of about 150 mg.)

Time after introduction (min.)	40	70	100	130	160	190	220
Activity in blood	0.04	0.02	0.05	0.10	0.12	0.11	0.13

amino-1-naphthyl) phosphate, should be investigated from this point of view.

Naphthylamine or its metabolites appear very rapidly in the blood and bile of the dog and the cat (Figs. 1, 2) after intraperitoneal injection, their fate being very similar to that observed in rabbits (Somerville *et al.* 1956). The transfer to the bile from the blood is very rapid and the active transport across the barrier separating the blood and biliary systems is striking, particularly in the cat, where the concentration in the bile is more than a hundred times that in the blood.

The behaviour of the metabolites of naphthylamine in the blood of guinea pigs (Table 2) is similar to that in rats (Somerville *et al.* 1956), in that the active material is detectable in the blood cells long after it has disappeared from the plasma. There is the difference that the fraction of the dose found in the blood cells of the guinea pig within the first few days is much smaller than in the rat, and it does not decrease exponentially with time, but in a slower, more irregular manner. The results for the dog (Table 3) are probably comparable with those in the rabbit (Somerville *et al.* 1956), for, although the individual counting rates in samples of plasma were low in the later stages, it appears that the active material was present in the plasma as long as it was in the cells.

The results in Table 4 confirm the previous observations that, after intraperitoneal injection of active naphthylamine in the rat, there is considerable retention of activity in the blood cells after it has disappeared from the plasma, but, in addition, they show that, at 13 or 20 days, practically all the activity present in the blood was in the cell contents, not in the cell membranes. Since the average life of red cells in the rat is 45–50 days (Burwell, Brickley & Finch, 1953), the great majority of cells present at 13 or 20 days after injection would have been present at the time of injection; the data in Table 4 are therefore held to support the view that the activity in the cell contents is due to the passage of the active material across the cell membrane, with subsequent attachment to one or more of the constituents of the contents. This attachment must be strong to prevent the leaching out of the material into the plasma, or its removal by repeated washing of the cells in sodium citrate solution. Whatever the combination may be, it persists after the plasma and membrane activities have practically disappeared, which is in marked contrast with the behaviour of the liver carcinogen, 4-dimethylaminoazobenzene, which is also strongly bound to the blood cells in the rat but is mostly attached to the membranes (Zischka, Karrer, Hromotka & Broda, 1954). It might have been expected that, similarly, the naphthylamine would be attached to the lipid cell membrane, but it

is bound to the cell contents. This may be evidence of the binding of a carcinogen to protein which is envisaged as the primary step in chemical carcinogenesis in the protein-deletion hypothesis (Miller & Miller, 1953).

In the clinical problem of occupational bladder tumours, the normal routes of absorption of the carcinogen have been variously held to be by the respiratory tract, by the skin or by both. Our findings recorded in Fig. 3 and Table 5 and the figures quoted for the dog show that both in the rat and the dog naphthylamine penetrates the skin in a short time. The concentration in the blood of the rats and the amounts found in their urine, in comparison with corresponding figures for animals which received naphthylamine by intraperitoneal injection, indicate that at least one-fifth of the dose applied to the skin was absorbed. It is more difficult to estimate the fraction absorbed by the dog, but it is probably of the order of a tenth. If human skin is penetrated with equal ease these quantities are of considerable significance in relation to the intense exposures suffered by workers in their employment 20 or more years ago.

SUMMARY

1. After the intraperitoneal injection of single doses of 2-[8-¹⁴C]naphthylamine into the dog, rabbit, guinea pig, rat and mouse (approx. 1 mg./kg. body wt.) the dog excretes only about 3 % of the radioactive material in the faeces, the guinea pig, rabbit and mouse about 10–15 % and the rat almost 30 %. The amount in urine varied from 62 to 90 %.

The small amount excreted by the dog in the faeces is accounted for by low excretion in the bile.

2. Paper chromatograms of urines show the differences in metabolism of 2-[8-¹⁴C]naphthylamine in these species.

3. The concentrations of radioactivity in the bile and in the blood of a dog and of a cat, after intraperitoneal injection, vary in a similar manner. The concentration at a given time is very much higher in the bile than in the blood.

4. Radioactivity is detectable in the blood of dogs and guinea pigs for several weeks after a single injection. In the dog the activity is shared between plasma and cells, but in the guinea pig after 3 weeks the activity is entirely in the cells.

5. The persistent activity in the blood of rats, after a single injection, is entirely in the contents of the cells, not in the plasma (previously shown) or in the red-cell envelope.

6. 2-[8-¹⁴C]Naphthylamine is rapidly absorbed through the skin of the rat and the dog, being readily recognizable in the blood within a short time after application to the skin.

7. 2-[8-¹⁴C]Naphthylamine is absorbed from the bladder into the blood of a cat to a notable extent, but 2-amino-1-[8-¹⁴C]naphthyl hydrogen sulphate is absorbed very much less readily.

We wish to thank Dr D. Manson, Institute of Cancer Research, Pollard's Wood, for advice on the extraction of the metabolic 2-amino-1-naphthyl hydrogen sulphate from dog urine. We are indebted to Mrs M. E. Ross, Miss P. A. Melhuish, Mrs R. Coombes and Mr R. G. Harrison for technical assistance.

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Biochem. J. (1960) **77**, 516

A Graphical Method for the Rapid Determination of Sedimentation Coefficients

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(Received 27 April 1960)

The calculation of sedimentation coefficients by the usual methods, though not particularly difficult, can be time-consuming, particularly if the systems examined in the analytical ultracentrifuge are complex. Various aids to this calculation, such as tabulated values of the various constants required, are in general use, but these save only a small amount of the mathematics required. The procedure described in this paper completely eliminates all calculation, and also serves as a convenient means for recording the data. In addition, the sedimentation coefficients obtained are based on an assessment of all the data available for each run. A system which can achieve this degree of simplification requires preparation, but it is hoped that this paper will enable this to be done with the least possible effort on the part of the user.

PRINCIPLE OF THE METHOD

The determination of a sedimentation coefficient (S) depends upon the solution of the equation

$$S = \frac{\ln(x_2/x_1)}{\omega^2(t_2 - t_1)}, \quad (1)$$

where x is the distance of a boundary from the axis of a rotor rotating with an angular velocity of ω at time t (Svedberg & Pedersen, 1940).

Equation (1) shows that a graph of $\ln x$ against t , for a substance having a constant sedimentation coefficient, is a straight line having a slope which is a function of ω^2 and S . Consequently, if x is plotted on logarithmic graph paper, S may be read off this curve by means of a protractor calibrated in terms of rev./min. against S .

Both the graph paper and the protractors have been designed for use with the Spinco Model E ultracentrifuge (Beckman/Spinco Division, Palo Alto, Calif., U.S.A.), but there is no reason why similar apparatus should not be employed with other ultracentrifuges. This type of ultracentrifuge has a number of fixed speeds and fixed intervals between photographic exposures, and the number of possible combinations is very large, so that a certain amount of selection has had to be made. The speeds covered are from 21 740 to 59 780 rev./min. and times from 4 to 246 min., which covers adequately all S values from 1 to 250s, and it is possible to extend this range considerably, as is explained below.

APPARATUS

The graph paper. Graph paper covering the range required is not available commercially at present, but it may be prepared fairly simply by one of several methods. It is conveniently included as part of a special data sheet, which