Metabolism of Bladder Carcinogens

2. THE METABOLIC PATH OF 2-[8-¹⁴C]NAPHTHYLAMINE IN THE RABBIT AND IN THE RAT*

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In a previous paper (Henson, Somerville, Farquharson & Goldblatt, 1954) we reported the results of an investigation into the distribution of 2-[8-¹⁴C]naphthylamine or its metabolites in the rat, following intraperitoneal injection. We now present the results of a similar study on rabbits, together with some additional results on rats.

MATERIALS AND METHODS

These were, with minor modifications, as previously described. Rabbits weighed from 1 to 2.7 kg.

Measurement of ¹⁴C. The correction for the dead time of a Geiger counter capacitatively coupled to a square-pulse-quenching unit proposed by Wyard (1953) has been found to give a slightly better agreement at high counting rates than the correction previously used (Henson, 1953). Recalculation on this basis reduced the measured efficiency of the counters from 72.1 to 70.9 % and the activity of the naphthylamine as assayed by the wet-oxidation method from $2.751 \pm 0.014 \times 10^7$ to $2.719 \pm 0.013 \times 10^7$ counts/min./mg.

Oxidation techniques. The method of preparing CO₂ from animal tissues for measurement in Geiger counters, which had been developed with rats, was unsatisfactory with most rabbit tissues. An impurity in the gas spoiled the counter characteristics, giving a slow rise to a plateau of high slope. This difficulty has been met by other workers using the method; it is apparently due to the production from protein of oxides of nitrogen which can be removed by exposing the gas to hot reduced copper (Bradley, Holloway & McFarlane, 1954; Glascock, 1954). We have introduced this step into our routine preparation of CO₂ from rabbit tissues and usually obtain satisfactory plateaux. We have not encountered this difficulty with any rat tissues, but almost all counter fillings from rabbit tissues have been unsatisfactory without the extra purification.

Anaesthesia. For the experiments in which bile was collected from cannulae inserted in the common bile duct, anaesthesia was initially produced by intraperitoneal injection of Nembutal (50 mg./kg. of body wt.) and was maintained throughout the experiment with cyclopropane-oxygen in rabbits and ether in rats. cycloPropane is liable

to produce respiratory depression and sudden failure in rabbits, and hence the adjustment of the ratio of cyclopropane to oxygen requires careful attention. The change from quiet even breathing to shallow, rapid and Cheyne-Stokes type is often very sudden.

RESULTS

Activity in the blood of rabbits after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine. The level of activity in the blood during the first few hours after injection was measured and the results for three normal rabbits (1, 2 and 3i), and for one with its bile duct ligated (7), are shown in Fig. 1. Samples of blood withdrawn from the ear were dried and weighed. The total activity in the animal's blood was calculated on the assumption that the blood lost 80 % of its weight on drying and that the weight of whole blood was 5 % of the body weight.

Partition of activity between blood plasma and blood cells in rabbits and rats. Four rabbits were injected intraperitoneally with approximately 1 mg. of 2-[8-¹⁴C]naphthylamine. Blood withdrawn at

Fig. 1. Activity in blood of rabbits after intraperitoneal injection of approx. 1 mg. of 2-[8-¹⁴C]naphthylamine. ●, ○, x, Normal rabbits 1, 2, 3i; △, rabbit 7 with ligated bile duct.

* Part 1: Henson, Somerville, Farquharson & Goldblatt (1954).

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various times from the ear was centrifuged and samples of plasma and cells (unwashed) were dried and weighed. The activities are shown in Table 1.

Washing the cells obtained 26 hr. after injection of the dose with four equal volumes of isotonic citrate removed 3.3, 2.2 and 2%, respectively, of the activity, but blood cells obtained from three rabbits 2 hr. after injection contained more loosely bound material, for 30–40% of the activity was removed in one citrate wash. Results for six rats are shown in Table 2. When rats 22 and 26 were killed the activity of the marrow from the femur of rat 22 was 0.3 count/min./mg. and of rat 26, none. Corresponding values for activity in blood cells were 13.6 and 10.0 counts/min./mg. The high level maintained in the blood cells of the rat is therefore not due to the continual release of new labelled cells from the marrow.

Excretion of radioactivity from rabbits after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine. The percentage of the dose excreted in the urine and faeces in three experiments is shown in Table 3. In the second experiment with rabbit 3 ii a collar was fitted to prevent refection of faeces in order to provide a better comparison with the results in rats.

Table 4 shows the distribution of the radioactive material in the excreta and various organs at different times after administration for eight rabbits, in three of which the bile ducts had been previously ligated. The effect of ligating the bile duct is much less noticeable than in rats. The reduction of the amount in the gastro-intestinal tract and faeces is not so marked, and it appears

Table 1. *Distribution of activity between blood plasma and cells in rabbits after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Results are expressed as counts/min./mg. dry wt. Doses: rabbits 8 and 9, 2.898×10^7 , rabbit 10, 3.059×10^7 , rabbit 11, 2.402×10^7 counts/min. Sample weights were such that the minimum detectable counting rate of 3 counts/min. above background was given by a sample having an activity of 0.2–0.5 count/min./mg. Samples having counting rates below 3 counts/min. are reported as zero.

Time after injection (hr.)	Rabbit 10		Rabbit 11	
	Plasma	Cells	Plasma	Cells
0.67	179.8	8.2	—	—
1.00	—	—	259.0	5.9
5.00	21.7	2.5	24.0	1.7
22.00	—	—	2.0	1.1
26.00	2.5	2.7	2.2	1.0

Time after injection (days)	Rabbit 8		Rabbit 9	
	Plasma	Cells	Plasma	Cells
1	5.6	0.5	6.2	0.4
4	7.2	0	2.3	0.6
7	2.7	0	1.9	0.4
11	1.9	0.3	0.9	0.5
18	4.9	0.4	2.8	1.1
25	0	0.2	0	0.7
33	0	0	0.6	0
40	1.0	0.2	0	0.3
42	0.3	0	—	—
47	—	—	7.5	1.3
54	—	—	1.7	0.4
61	—	—	0.5	0.2
67	—	—	0	0
69	—	—	0	0

Table 2. *Concentration in blood plasma and cells of rats after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Results are expressed as counts/min./mg. dry wt. Dose to each rat 3.037×10^7 counts/min. Last entries pertain to blood taken at death, those in parentheses to washed cells.

Time (days)	Rat 22		Rat 23		Rat 24		Rat 25		Rat 26		Rat 27	
	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells
1	45.3	56.4	36.6	88.3	51.8	65.2	43.7	88.8	51.7	50.5	45.6	88.3
4	12.5	45.6	8.9	75.0	6.7	60.0	9.2	68.4	8.3	44.9	10.1	69.1
7	48.4	41.5	7.6	62.0	8.6	48.0	41.6	54.9	3.4	38.3	1.6	48.7
11	1.4	32.3	8.7	40.2	3.0	37.1	3.9	42.0	2.1	32.7	2.0	33.1
14	—	28.5	2.4	20.8	3.2	29.9	0.6	25.8	1.0	40.4	—	15.9
19	—	—	2.7	12.8	9.7	18.9	0	18.3	1.6	19.0	1.0	19.1
25	—	—	2.0	7.3	0	12.8	0.7	10.3	—	—	0	9.2
26	0.5	13.6 (12.5)	—	—	—	—	—	—	—	—	—	—
28	—	—	0	5.4	0	11.5	0	8.1	0.4	10.0	0	6.6
35	—	—	0	2.8	0	6.9	0	3.5	—	—	0	4.8
42	—	—	0	2.9	0	1.5	0	1.3 (1.2)	—	—	0	2.2
47	—	—	0	0.6	—	—	—	—	—	—	0	1.4
48	—	—	—	—	0.4	1.0 (0.9)	—	—	—	—	—	—
53	—	—	0.5	0.24 (0.18)	—	—	—	—	—	—	—	—
56	—	—	—	—	—	—	—	—	—	—	0	0.4 (0.4)

that a substantial fraction of the dose reaching the gastro-intestinal tract does so by other routes than the bile. In order to investigate this the bile ducts of three rabbits were cannulated and bile was collected continuously after intraperitoneal injection of naphthylamine. The rates at which rabbits 17 and 20 secreted bile, 46.2 ml. in 5.5 hr. and 47.4 ml. in 4 hr. respectively, were of the same order as those observed by Archdeacon, Danforth & Dummit (1954). For rabbit 19 the rate was

9.8 ml. in 4.5 hr. Results are summarized in Table 5 and Figs. 2 and 3. The ratio of the concentration of activity in the bile to that in the blood (values taken from the smoothed curves in Fig. 3) decreased from 7.7 at 30 min. to 2.8 at 3.5 hr. in rabbit 19, but in rabbit 20 the ratio rose from 17.8 to 32.1 at the same times. These experiments confirm those results obtained by ligation of the bile duct in that the activity contained in the bile does not represent the great majority of that which reaches the gastro-intestinal tract, as it does in the rat. Comparable figures for a rat with a cannulated bile duct are shown in Table 5.

The bile from rabbit 19 was filtered through a semi-permeable cellulose membrane under reduced pressure. The residue on the filter was suspended in water and again filtered. The filtrates contained all the radioactivity of the original bile and gave no biuret reaction for protein, from which it is concluded that the metabolites are not at this stage firmly bound to protein.

Table 3. *Percentage of dose excreted in urine and faeces of rabbits after intraperitoneal injection of 2-[8-¹⁴C]naphthylamine*

Dose approx. 1 mg. Rabbit 3ii was fitted with a collar.

Rabbit no.	Time after injection (days)	Urine (% of dose)	Faeces (% of dose)
1	1.3	81.7	7.8
3i	18	89.9	7.4
3ii	17	82.4	13.3

Table 4. *Percentage of dose present in tissues and excreta of rabbits after intraperitoneal injection of approx. 1 mg. of 2-[8-¹⁴C]naphthylamine*

Time after injection ...	1 hr.	2 hr.				26 hr.		
Animal no. ...	6	4	5	12*	14*	10	11	7*
Urine	39.5	62.4	55.9	55.1	40.0	77.4	82.6	90.2
Faeces	—	0.003	0.3	—	—	0.6	8.7	0.03
Stomach	2.3	1.8	3.3	2.4	4.6	2.6	0.6	1.1
Small intestine	21.7	4.0	2.0	3.3	3.8	0.4	0.2	1.9
Large intestine	2.1	11.7	17.1	12.3	2.6	4.7	7.3	1.1
(Gastro-intestinal tract and faeces)	(26.1)	(17.5)	(22.7)	(18.0)	(11.0)	(8.3)	(16.8)	(4.1)
Liver	1.6	1.4	1.7	1.9	2.5	0.2	0.2	0.8
Kidneys	4.2	1.6	2.0	1.1	5.7	0.03	0.06	0.9
Carcass	—	—	—	—	21.7	1	—	4.0
Total	71.4†	82.9†	82.3†	76.1†	81.3‡	86.9	99.6†	100.0

* Bile duct previously ligated.

† Excluding carcass.

‡ Includes bile 0.4.

Table 5. *Percentage of dose present in tissues and excreta of rabbits and a rat with cannulated bile ducts after injection of 2-[8-¹⁴C]naphthylamine*

Anaesthesia was maintained throughout the experiment. Rabbit 19 was starved for 16 hr. before the experiment. Dose approx. 1 mg.

Time after injection (hr.)	Rabbits			Rat
	3.25	4.5	4.0	
Animal no. ...	17	19	20	29
Bile collected	8.3	3.5	17.5	34.4
Bile in gall bladder	—	1.5	0.5	—
Gastro-intestinal tract and contents	4.4	7.8	4.0	11.0
Urine	42.0*	23.2	58.8	11.8
Bladder	1.25	0.5	2.6	0.2
Kidneys	7.2	32.9	2.3	5.2
Liver	2.4	2.8	0.7	5.1
Carcass	19.5	17.6	5.6	27.2
Recovery	85.0	89.8	92.0	94.9

* Some urine was lost.

Fig. 2. Flow of bile and excretion of dose in bile of rabbit 17 after intraperitoneal injection of approx. 1 mg. of 2-[8- 14 C]naphthylamine. $\circ$, Rate of bile flow; $\bullet$, rate of excretion of dose in bile.

Fig. 3. Concentration of activity in blood and bile of rabbits after intraperitoneal injection of 2-[8- 14 C]naphthylamine. Rabbit 19: dose 2.156×10^7 counts/min.; $\circ$, blood; $\times$, bile. Rabbit 20: dose 3.765×10^7 counts/min.; $\triangle$, blood; $\bullet$, bile. The points on the bile curves represent the mean concentration in bile collected over periods of 15 or 30 min.

DISCUSSION

The variation in concentration of activity in the blood of normal rabbits follows a similar course to that in rats, rising to a maximum in 0.5–1 hr. (Fig. 1, rabbits 1, 2 and 3i), but the maximum is not so well defined and the rate of fall is lower. The effect of ligating the bile duct (rabbit 7) is less than in the rat, for apart from rising to a somewhat higher level the blood concentration follows the normal pattern, whereas in the rat the maximum is broadened and the fall delayed (Henson *et al.* 1954, Table 1). This is a reflexion of the fact that in the rabbit only a small fraction of the dose is excreted in the bile.

Table 1 shows that in the first few hours most of the activity in the blood is in the plasma. After one day the plasma concentration (on a dry-weight

basis) still tends to remain higher than that in the cells, both then falling to below the level of detectability over a period of several weeks with the plasma concentration remaining the higher. This is very different from the findings with the rat (Henson *et al.* 1954, Table 3), where after periods of 10 and 15 days the activity is almost entirely in the blood cells. The further results on rats reported in Table 2 confirm this difference between the two species. After one day all six rats have comparable dry-weight concentrations in plasma and cells, but that in the plasma then decreases rapidly. The cell activity decreases much more slowly so that after 11 days the blood activity is almost wholly in the cells. Washing the cells removes very little activity. The decline of activity in the cells can be reasonably approximated by exponential curves having time constants of 17.6, 9.0, 14.5, 10.3, 16.5, 10.4 days for rats 22 to 27 respectively. If the cells were initially labelled with active material which remained fixed until the cell reached the end of its life and was then removed from the blood, a linear decrease would be expected. The average life of red cells in a rat has been reported to be 45–50 days (Burwell, Brickley & Finch, 1953); this would require a slower decrease than we have observed. The exponential decrease probably means that the activity is bound to a cell constituent which is in dynamic equilibrium, the time constant of the exponential representing the mean life of this constituent. We have as yet no explanation of the sudden rise in activity in the plasma of rats 22 and 25 at 7 days and rat 24 at 19 days. The effect in rabbit 9 at 47 days is presumably due to the same cause.

Rabbits 8 and 9 were killed 42 days and 69 days respectively after injection when the activity in the blood had reached the limit of detectability. At this stage the activities in the kidneys, liver and bone of rabbit 8 were just on the limit of detectability with none detectable in the bladder and bone marrow. There was no detectable activity in any organ of rabbit 9. Thus in the rabbit, as in the rat, naphthylamine or its metabolite is retained in the blood at least as long as in any other part of the body.

The initial excretion of the active material in the urine is much more rapid in rabbits than in rats. The former excrete over 50% of the dose in 2 hr. (Table 4), and the latter about 22% (Henson *et al.* 1954, Table 5). The total fraction excreted in the urine is also higher, over 80% compared with 65% in the rat (Table 3, and Henson *et al.* 1954, Fig. 1) and the amount reaching the gut is correspondingly less. The figures in Table 4 for the total in the faeces and gastro-intestinal tract show for normal animals a general decline with time which indicates a reabsorption of the material from the gut, as in the rat.

The proportion of the dose excreted in the bile of the rabbit is small, as shown by the slight effect of ligating the bile duct on the contents of the gastrointestinal tract and faeces (Table 4), and it appears that a comparable amount reaches the gut by other routes. In the rat a much larger percentage reaches the gut, the great majority being carried in the bile. These conclusions were confirmed by the experiments with cannulated animals (Table 5), which show that in rabbits active material still reached the gut from extra-biliary sources in amounts generally of the same order as that excreted in the bile. In the rat 11% of the dose reached the gut in 1.75 hr. but over three times as much was found in the bile.

Naphthylamine or its metabolites appear very rapidly in the bile (Fig. 2), the rate of excretion reaching a maximum within 15 min. in rabbit 17. Apart from a somewhat sharper maximum the curve closely resembles those showing variation of concentration in the blood (Figs. 1 and 3). Within the accuracy of our measurements the concentration in the bile does not lag behind that in the blood. The transfer of the material from the blood to the bile is not merely a matter of diffusion through a membrane, for the concentration in the bile is much higher than in the blood, strikingly so in the case of rabbit 20, despite the fact that the actual volume of bile excreted by this animal was very high.

SUMMARY

1. Rabbits injected intraperitoneally with 2-[8-¹⁴C]naphthylamine (approx. 1 mg.) retain radioactivity in the blood for several weeks. This activity is shared between the plasma and cells. In rats, activity is detectable in the blood for about

the same length of time, but after 11 days it is entirely in the cells.

2. In one day after intraperitoneal injection of approximately 1 mg. of 2-[8-¹⁴C]naphthylamine in rabbits about 90% of the dose is excreted in the urine and faeces. The initial excretion in the urine is very rapid (over 50% of the dose in 2 hr., compared with 22% for rats). Total urinary excretion in the rabbit is about 80%.

3. Excretion of radioactive material into the gut of rabbits, both by the biliary route and otherwise, is demonstrated. The total amount reaching the gut is much less than in rats, mainly because excretion in the bile is much lower. Reabsorption of active material from the gut is shown to occur.

4. The concentration of radioactivity in the bile and the blood of rabbits varies in a closely similar manner. The actual concentration in the bile at a given time is considerably higher than in the blood and may exceed it from 3 to over 30 times in anaesthetized rabbits with cannulated common bile ducts.

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The Enzymic Synthesis of Steroid Sulphates

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Soluble enzyme preparations from rat liver have been known for some time to be capable of synthesizing aryl sulphates from the appropriate phenol and sulphate ions in the presence of adenosine triphosphate (Bernstein & McGilvery, 1952a; DeMeio, 1952). There had been no investigation of the synthesis of steroid sulphates by such enzymes until very recently, when DeMeio & Lewycka (1955) were able to demonstrate the probable synthesis thereby of dehydroepiandrosterone sul-

phate (DHAS), although the product was not characterized. The synthesis of such steroid sulphates is of particular interest as these compounds must play a part in the metabolism of steroids, and their synthesis is probably more representative of the true function of the enzyme system involved than is the commonly studied formation of the sulphates of toxic phenols. Furthermore, the molluscan enzyme hydrolysing these steroid sulphates, steroid sulphatase, showed