The Metabolism of Isolated Rat-Liver Nuclei during Chemical Carcinogenesis

2. 2-ACETAMIDOFLUORENE, α -NAPHTHYL ISOTHIOCYANATE AND 2',4'-DIMETHYL-4-DIMETHYLAMINOAZOBENZENE*

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Rat-liver nuclei have been studied during the 6-month induction period of liver cancer produced by feeding either thioacetamide or *p*-dimethylaminoazobenzene to rats (Rees & Rowland, 1961*b*). An increase in the amount of chemical constituents of the nuclei was observed with both carcinogens, but synthesis by isolated nuclei of RNA and protein, as measured by the incorporation of ^{14}C -labelled metabolites, was affected differently by the two carcinogens. In order to determine whether the nuclear changes are related to the carcinogenic process, the studies have been continued with 2-acetamidofluorene, α -naphthyl isothiocyanate and 2',4'-dimethyl-4-dimethylaminoazobenzene.

2-Acetamidofluorene was shown by Wilson, DeEds & Cox (1941) to produce tumours in various organs including the liver, when fed to rats. The histological changes in the livers of rats fed 2-acetamidofluorene were described in detail by Firminger (1955). Nuclear changes studied during the 20 weeks of feeding 2-acetamidofluorene to rats are described in this paper.

Since 2-acetamidofluorene and the two carcinogens studied by Rees & Rowland (1961*b*) produce bile-duct proliferation at early stages during the feeding period, it is of interest to see whether biochemical nuclear changes can be brought about by the feeding of a substance that causes bile-duct proliferation without the subsequent development of liver cancer. Such a substance is α -naphthyl isothiocyanate, the effects of which are described by Lopez & Mazzanti (1955). Nuclei have been isolated from livers of rats fed α -naphthyl isothiocyanate for 3, 6 and 9 weeks and examined for changes in chemical composition and synthetic ability.

In order to check that the nuclear changes obtained during the feeding of *p*-dimethylaminoazobenzene to rats (Rees & Rowland, 1961*b*) are related to the hepatocarcinogenic action of the dye, the 2',4'-dimethyl derivative, shown by Miller &

Miller (1948) to be non-carcinogenic, has been fed to rats for 20 weeks, and their liver nuclei have been isolated and studied at intervals during the feeding period.

As in the thioacetamide and *p*-dimethylaminoazobenzene experiments, nuclear changes have been examined in relation to histological changes and a close correlation has been observed.

EXPERIMENTAL

Nuclear preparations. Nuclei were isolated in 0.25M-sucrose as described by Rees & Rowland (1961*a*).

Radioactive substances. [$2\text{-}^{14}\text{C}$]Glycine, [$6\text{-}^{14}\text{C}$]orotic acid and [$8\text{-}^{14}\text{C}$]adenine hemisulphate were obtained from The Radiochemical Centre, Amersham, Bucks.

For incorporation of [$6\text{-}^{14}\text{C}$]orotic acid into RNA and [$8\text{-}^{14}\text{C}$]adenine into nucleic acids the system used was: sodium phosphate buffer, pH 7.4, 20 mM; glucose, 12 mM; NaCl, 10 mM; $1\text{ }\mu\text{C}$ of [^{14}C]adenine or [^{14}C]orotic acid; 1.5 ml. of nuclear suspension in 0.25M-sucrose; water to 2.5 ml. The temperature was 30° and the incubation time was 90 min. The reaction was stopped by the addition of 2.0 ml. of ice-cold 10% (w/v) HClO_4 . The precipitate was washed four times with 2.0 ml. of ice-cold 2% (w/v) HClO_4 , twice with ethanol and twice with ether.

For incorporation of [$2\text{-}^{14}\text{C}$]glycine into nuclear protein the system used was: sodium phosphate buffer, pH 7.4, 20 mM; NaCl, 20 mM; [^{14}C]glycine, $1\text{ }\mu\text{C}$; 1.5 ml. of nuclear suspension in 0.25M-sucrose; water to 2.5 ml. The temperature was 38° and the time of incubation was 90 min. The reaction was stopped by the addition of 2.0 ml. of 20% trichloroacetic acid. The precipitate was extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 20 min. at 90° . The protein residue was then washed successively with 5 ml. of 5% trichloroacetic acid containing 1% (w/v) glycine and 5 ml. of 5% trichloroacetic acid, then twice with ethanol and twice with ether. The protein residue was dried at room temperature.

The methods for determining radioactivity are as described by Rees & Rowland (1961*a*).

Chemical determinations. Nuclear counts, nitrogen, RNA, DNA and phospholipids were determined as described by Rees & Rowland (1961*a*).

Animals. Male Norwegian hooded rats bred from the same colony were used. Rats were put on the diets when their body weights reached 140–170 g. The basic diet was

* Part 1: Rees & Rowland (1961*b*).

Medical Research Council 41B meal (Bruce & Parkes, 1946) fed *ad lib*. Additions to diets were as follows: 2-acetamidofluorene, 500 mg. was mixed with 1 kg. of meal; α -naphthyl isothiocyanate, 1 g. was mixed with 1 kg. of meal; 2',4'-dimethyl-4-dimethylaminoazobenzene, 600 mg. suspended in 20 ml. of ethanol was mixed with 1 kg. of meal. The dye was prepared by coupling the diazonium salt of 2,4-xyldine with dimethylaniline as described by Miller & Miller (1948). Water was given *ad lib*.

RESULTS

Histological changes

2-Acetamidofluorene. Three main stages were observed on histological examination of the livers of rats fed with 2-acetamidofluorene for 20 weeks. In the first stage (1–5 weeks) the livers appeared normal apart from the presence of a few chronic inflammatory cells in the region of the portal spaces. Towards the end of this stage, however, signs of bile-duct proliferation were beginning to appear. The second stage (6–16 weeks) showed progressively severe bile-duct proliferation with a maximum severity at approximately 14 weeks. This was accompanied by pseudo-lobulation of the liver, many chronic inflammatory cells and progressively marked numbers of enlarged parenchymal cell nuclei. During the third stage (16–20 weeks) the bile-duct proliferation had passed its maximum and rapidly disappeared, to be replaced by areas of cellular regeneration. Animals killed at 26 weeks were found to exhibit large tumours which, on microscopic examination, were primary hepatocarcinomas.

α -Naphthyl isothiocyanate. After 3 weeks of feeding, there was very marked bile-duct proliferation present resulting in the breaking up of the liver pattern in a pseudo-cirrhotic manner. Apart from this, the liver parenchymal cells appeared normal. A similar histological picture was obtained after 6 and 9 weeks' feeding but of a much less severe nature, the bile-duct proliferation now appearing moderate. At 16 weeks moderate bile-duct proliferation was still present.

2',4'-Dimethyl-4-dimethylaminoazobenzene. Livers of rats fed with this compound were histologically normal over the 20 weeks of feeding.

Chemical composition

2-Acetamidofluorene. During the first 3 weeks of feeding, no change in nuclear numbers was observed. In the following weeks, however, numbers increased with a maximum at 10 weeks. After this, values appeared to return to control levels (Table 1). The nuclear nitrogen rose more rapidly, a 50% increase being obtained after 2 weeks and, although a maximum was reached at 10–14 weeks, values did not return to normal

(Table 1). The difference in the rats of increase and decrease of nuclear nitrogen and nuclear numbers is demonstrated in the rise, fall, and rise again of nitrogen per nucleus.

DNA content of nuclei during the feeding period rose rapidly (Table 2). After 1 week, a 25–30% increase was observed and values increased to 50% over control levels at 10–14 weeks. After this, values fell to levels 25% above control at 19 weeks. RNA phosphorus was also increased after 2 weeks of feeding and remained raised, thus following closely the changes in nuclear nitrogen (Table 2).

Phospholipid phosphorus increased slowly at first but by 10–14 weeks a 200–300% increase was observed. At 19 weeks the values had declined slightly (Table 3).

α -Naphthyl isothiocyanate. There was a rapid rise in nuclear numbers during the feeding of α -naphthyl isothiocyanate so that after 3 weeks the values were 35% above control levels. At 6 and 9 weeks, however, numbers declined (Table 4). Nuclear nitrogen was raised at 3 and 6 weeks but had returned to normal at 9 weeks (Table 4).

The DNA phosphorus was observed to increase rapidly (Table 5). At 3 weeks this increase, in terms of liver weight, was 60% over the control level and, hence, showed an increased DNA per nucleus. At 6 and 9 weeks, however, the values were closely related to nuclear numbers. RNA was also increased at 3 and 6 weeks, but the values per nucleus were unaltered (Table 5).

Phospholipid phosphorus was increased at 3 and 6 weeks in terms of liver weight, and was normal

Table 1. *Nuclear numbers and total nuclear nitrogen of liver from rats given 2-acetamidofluorene*

Weeks of feeding	Nuclei/g. wet wt. of liver (millions)	Total nuclear nitrogen	
		(mg./g. wet wt. of liver)	(μ g./nucleus)
Control	116 $\pm$ 10.2	1.58 $\pm$ 0.04	13.7 $\pm$ 0.91
1	118	1.62	13.8
	115	1.55	13.4
2	87	1.93	22.1
	111	2.34	21.1
3	105	2.15	20.6
	150	2.22	14.8
6	130	1.91	14.7
	138	1.82	13.2
10	257	2.72	11.6
	216	3.06	14.2
14	132	2.45	18.6
	155	3.62	23.2
19	121	2.42	20.0
	116	1.84	15.9

Table 2. *Nucleic acid phosphorus in liver nuclei from rats given 2-acetamidofluorene*

Control values are the means of 18 animals killed at intervals during the feeding period $\pm$ s.e. Other values are from individual rats.

Weeks of feeding	Phosphorus ($\mu\text{g./g. wet wt. of liver}$)		Phosphorus ($\mu\text{g./mg. of total nuclear nitrogen}$)		Phosphorus ($\mu\text{g./nucleus}$)	
	DNA	RNA	DNA	RNA	DNA	RNA
Control	115 $\pm$ 5.9	69.0 $\pm$ 2.5	72.8 $\pm$ 2.9	42.3 $\pm$ 1.4	0.99 $\pm$ 0.05	0.575 $\pm$ 0.04
1	140	66	87	41	1.19	0.56
	147	68	95	44	1.27	0.60
2	147	79	63	43	1.68	0.91
	149	100	79	41	1.34	0.99
3	164	67	75	31	1.10	0.54
	151	80	70	36	1.45	0.65
6	142	70	75	38	1.09	0.54
	158	73	87	41	1.15	0.53
10	154	107	51	35	0.60	0.41
	162	102	60	37	0.76	0.47
14	152	123	63	52	1.14	0.94
	176	164	49	46	1.12	1.04
19	137	95	57	40	1.13	0.79
	147	100	80	51	1.28	0.82

Table 3. *Phospholipid phosphorus of nuclei isolated from livers of rats given 2-acetamidofluorene*

Control values are the means of 18 animals killed at intervals during the feeding period $\pm$ s.e. Other values are from individual rats.

Weeks of feeding	Phospholipid phosphorus		
	($\mu\text{g./g. wet wt. of liver}$)	($\mu\text{g./mg. of nuclear N}$)	($\mu\text{g./nucleus}$)
Control	60.5 $\pm$ 3.6	38.1 $\pm$ 1.0	0.525 $\pm$ 0.04
1	70	43	0.60
	43	28	0.37
2	77	33	0.88
	58	30	0.52
3	76	34	0.51
	73	34	0.71
6	96	50	0.74
	76	42	0.55
10	170	56	0.66
	155	57	0.72
14	118	49	0.89
	198	55	1.27
19	93	39	0.77
	89	46	0.73

Table 4. *Nuclear numbers and total nuclear nitrogen of liver from rats given α -naphthyl isothiocyanate*

Control values are the means of 18 animals killed at intervals during the feeding period $\pm$ s.e. Other values are from individual rats.

Weeks of feeding	Nuclei/g. wet wt. of liver (millions)	Total nuclear nitrogen	
		(mg./g. wet wt. of liver)	($\mu\text{g./nucleus}$)
Control	116 $\pm$ 10.2	1.58 $\pm$ 0.04	13.7 $\pm$ 0.91
3	150	1.71	11.4
	160	1.85	11.5
6	150	1.87	12.5
	145	2.00	13.8
9	120	1.41	11.7
	140	1.55	11.1

per nucleus (Table 6). At 9 weeks, however, values fell below control levels.

2',4'-Dimethyl-4-dimethylaminoazobenzene. No change was observed in the composition of nuclei from the livers of rats fed with this compound for 20 weeks.

Incorporation experiments

As with previous experiments (Rees & Rowland, 1961b), incorporation of [^{14}C]glycine into nuclear protein, [^{14}C]orotic acid into nuclear RNA and [^{14}C]adenine into nuclear nucleic acids *in vitro* was studied during the feeding of the various substances to rats. Orotic acid is essentially a specific precursor of RNA in the livers of adult animals (Hurlbert & Potter, 1952; Hecht & Potter, 1956). Since the pattern of change obtained in adenine incorporation parallels that of orotic acid, it is considered that the results obtained in this paper for adenine also represent RNA synthesis rather than DNA synthesis. Where changes have been observed, results are expressed as a percentage increase or decrease over the corresponding control value.

2-Acetamidofluorene. Although there was no change in the incorporation of glycine, the levels of both orotic acid and adenine incorporation decreased during the feeding of 2-acetamidofluorene and remained low (Fig. 1).

α -Naphthyl isothiocyanate. Animals at 3 weeks showed a slight increase in glycine incorporation and a 30% decrease in the incorporation of orotic acid and adenine. These changes were, however, not maintained, and were indeed somewhat reversed at 9 weeks (Fig. 2).

2',4'-Dimethyl-4-dimethylaminoazobenzene. No change in incorporation was observed at any stage during the feeding of this substance.

Table 5. *Nucleic acid phosphorus in liver nuclei from rats given α -naphthyl isothiocyanate*

Control values are the means of 18 animals killed at intervals during the feeding period $\pm$ s.e. Other values are from individual rats.

Weeks of feeding	Phosphorus (μ g./g. wet wt. of liver)		Phosphorus (μ g./mg. of nuclear nitrogen)		Phosphorus (μ g./nucleus)	
	DNA	RNA	DNA	RNA	DNA	RNA
Control	115 $\pm$ 5.9	69.0 $\pm$ 2.5	72.8 $\pm$ 2.9	42.3 $\pm$ 1.4	0.99 $\pm$ 0.05	0.575 $\pm$ 0.04
3	183	79	107	46	1.22	0.53
	189	86	101	46	1.18	0.54
6	151	87	81	46	1.01	0.57
	141	79	71	40	0.98	0.54
9	138	64	98	45	1.15	0.53
	137	66	89	43	0.98	0.47

Table 6. *Phospholipid phosphorus of nuclei isolated from livers of rats given α -naphthyl isothiocyanate*

Control values are the means of 18 animals killed at intervals during the feeding period $\pm$ s.e. Other values are from individual rats.

Weeks of feeding	Phospholipid phosphorus		
	(μ g./g. wet wt. of liver)	(μ g./mg. of nuclear N)	(μ g./nucleus)
Control	60.5 $\pm$ 3.6	38.1 $\pm$ 1.0	0.525 $\pm$ 0.04
3	74	43	0.49
	84	45	0.52
6	78	42	0.52
	87	44	0.60
9	37	26	0.31
	42	27	0.30

Fig. 1. Incorporation *in vitro* of [14 C]adenine [●] and [14 C]orotic acid [○] into liver nuclei from rats given 2-acetamidofluorene. Percentage deviation was from controls. Mean control values (···) for 22 animals are (counts/min. corrected to infinite thickness): orotic acid, 172 ($\pm$ 14); adenine, 148 ($\pm$ 15). Each point represents the mean value from at least two animals.

DISCUSSION

The results obtained in this paper for the chemical composition of nuclear preparations during 2-acetamidofluorene feeding do not represent changes solely in nuclei of parenchymal cells, since the pro-

Fig. 2. Incorporation *in vitro* of [14 C]adenine [●], [14 C]orotic acid [○] and [14 C]glycine [+] into liver nuclei from rats given α -naphthyl isothiocyanate. Percentage deviation was from controls. Mean control values (···) for 22 animals are (counts/min. corrected to infinite thickness): orotic acid, 172 ($\pm$ 14); adenine, 148 ($\pm$ 15); glycine, 250 ($\pm$ 18). Each point represents the mean value from at least two animals.

portion of parenchymal cell nuclei to bile-duct cell nuclei changes during the feeding period. Thus when values of the constituents per nucleus fall (14 weeks), bile-duct proliferation is at its maximum and it is likely that there are large numbers of bile-duct-cell nuclei present in the preparations for analysis. At periods when there is little or no bile-duct proliferation (first and third histological stages), chemical constituents per nucleus are in-

creased. It seems likely, therefore, that increases in the amounts of constituents per parenchymal cell nucleus are a true manifestation of 2-acetamidofluorene carcinogenesis.

The changes observed in the incorporation experiments appear to contradict the findings of changes in chemical composition. If incorporation of precursors into RNA and protein by nuclei *in vitro* reflects synthesis *in vivo*, increased amounts of RNA and protein per nucleus would lead one to expect increased levels of incorporation. However, no such increase is observed and instead a decreased incorporation into RNA is obtained.

A similar result was obtained during thioacetamide carcinogenesis (Rees & Rowland, 1961*b*) and it was suggested that the presence of pycnotic nuclei may have a masking effect on increased incorporation by nuclei that were showing enlargement. Since no pycnosis was observed during 2-acetamidofluorene feeding, this possibility is ruled out and also throws some doubt on this explanation for the thioacetamide experiments. Another possible explanation for the discrepancy could be that RNA and protein breakdown are decreased more than RNA and protein synthesis with the result that there is a net increase in amounts of these substances in the nucleus.

With feeding α -naphthyl isothiocyanate, biochemical findings support the histological evidence that there is no effect on the liver parenchymal cells, but only on bile-duct cells. Thus increased amounts of substances in the nuclear preparations are correlated with the presence in the liver of severe bile-duct proliferation and, apart from DNA levels at 3 weeks, values for constituents per nucleus are not increased. The increased DNA probably reflects active cell division involved in the bile-duct proliferation occurring at this time. It appears therefore that with the feeding of α -naphthyl isothiocyanate there is no increase in chemical constituents of the parenchymal cell nuclei as observed with the feeding of the carcinogenic substances.

The decreased incorporation into RNA by nuclei from rats given α -naphthyl isothiocyanate for 3 weeks is similar to the changes observed with 2-acetamidofluorene feeding and with the feeding of thioacetamide (Rees & Rowland, 1961*b*). The changes were not, however, maintained and are probably of little significance.

The absence of both histological and biochemical change in the livers of rats fed with 2',4'-dimethyl-4-dimethylaminoazobenzene is in agreement with the findings of other workers (Miller & Miller, 1948, 1955). It may be concluded, therefore, that the nuclear changes observed during the feeding of *p*-dimethylaminoazobenzene (Rees & Rowland, 1961*b*) are a true manifestation of chemical

carcinogenesis and not connected solely with the ingestion of an azo dye.

The results of the present investigation and those of the previous work (Rees & Rowland, 1961*b*) may be summarized as follows. At a very early stage in the development of liver cancer induced by chemical means, the nuclei of the parenchymal cells show an increase in amounts of their chemical constituents, irrespective of the nature of the inducing agent. The incorporation experiments indicate that the mechanism for nuclear change differs according to the substance administered. With *p*-dimethylaminoazobenzene the increased nuclear RNA and protein appear to result from increased synthesis of these substances in the nucleus, whereas with thioacetamide and 2-acetamidofluorene it may be that there is a decreased breakdown of these components in the nucleus.

SUMMARY

1. Nuclei were isolated in 0.25*M*-sucrose from livers of normal rats and rats given 2-acetamidofluorene, α -naphthyl isothiocyanate or 2',4'-dimethyl-4-dimethylaminoazobenzene.

2. Nuclear counts, nuclear nitrogen, deoxyribonucleic acid and ribonucleic acid and phospholipids were determined at intervals during the feeding periods. Increased values of chemical constituents per parenchymal cell were obtained with feeding the carcinogen 2-acetamidofluorene but not with the other two substances which are non-carcinogenic.

3. The incorporation *in vitro* of [¹⁴C]glycine into nuclear protein, [¹⁴C]orotic acid into nuclear ribonucleic acid and [¹⁴C]adenine into nuclear nucleic acids was studied during the feeding periods. The only clear change obtained was a fall in the orotic acid and adenine incorporation during the feeding of 2-acetamidofluorene.

4. The conclusion is drawn that, whereas chemical hepatocarcinogenesis is always accompanied by an increased chemical composition of parenchymal cell nuclei, the mechanism for this increase varies according to the carcinogen administered.

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A Study of the Organic Material of Hen's-Egg Shell

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The general structure of the hen's-egg shell was reviewed by Simkiss & Tyler (1957). Surrounding the egg white are two shell membranes each composed of a network of fibres. The shell is attached to the outer of these membranes by the mammillae, which are roughly hemispherical knobs composed of large spherulite crystals of calcite. Each mammilla contains a core of organic matter and merges above into the main body of the shell. Although this is known as the spongy layer it is composed of a dense mass of calcite crystals bound together by a sparse organic matrix. The shell is pierced by funnel-shaped tubes, the pores, which start from the interstices between the mammillae, pass through the shell and open on the surface. Covering the surface of the shell and the mouths of the pores is a thin, tough cuticle. The structure of the shell is shown diagrammatically in Fig. 1.

Although the general structure of the shell is established, little work has been done on the

chemical composition of the organic components. Moran & Hale (1936) studied the shell membranes histologically and were able to separate each into a number of layers. The outer membrane, i.e. that next to the shell, consisted of three layers, the outermost of keratin fibres and the other two of mucin fibres, and the inner membrane consisted of two rather indistinct layers of keratin and mucin fibres. Partial amino acid analyses (Calvery, 1933; Jones & Mecham, 1944; Munks, Robinson, Beach & Williams, 1945), together with X-ray diffraction studies (Wolken, 1951), support the view that the membranes are composed largely of keratin.

Almquist (1934) investigated the matrix chemically and concluded that the protein was a collagen. From histochemical studies Simkiss & Tyler (1957) showed that the matrix consisted of a protein-acid polysaccharide complex. A re-appraisal of the results of the staining techniques (Simkiss, 1958) indicated also the presence of neutral sugar, particularly in the mammillary cores. The mammillae stained strongly for fat whereas only very faint staining was observed in the rest of the matrix.

Dickie (1848) suggested that the cuticle was a cellular epithelium over a basement membrane. Marshall & Cruickshank (1938) stated that it was porous, but otherwise structureless, and that it thickened over the mouths of the pores forming the cuticular plaques. Both Moran & Hale (1936) and Haines & Moran (1940) considered the cuticle to be mainly mucin, whereas Conrad & Scott (1938) suggested the possibility of its formation from egg white diffusing out of the egg and Romanoff & Romanoff (1949) believed that it was similar to the egg-shell membranes. The presence of protein containing many disulphide links and free sulphydryl groups and some evidence for the presence of phospholipid was shown by Simkiss (1958).

Fig. 1 Conventional diagram of a radial section of a hen's-egg shell to show the general structure.