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The Metabolism of Isolated Rat-Liver Nuclei during Chemical Carcinogenesis

THIOACETAMIDE AND *p*-DIMETHYLAMINOAZOBENZENE (BUTTER YELLOW)

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The feeding of thioacetamide to rats for 6 months or more results in the appearance of liver tumours (cholangiocarcinoma) (Fitzhugh & Nelson, 1948). Histological changes are, however, apparent in the liver after a few days of feeding (Gupta, 1956). The most striking of these early changes is the enlargement of the parenchymal cell nucleus and nucleolus. Preliminary studies on the nuclear fraction of liver homogenates after several weeks of thioacetamide feeding showed an altered chemical composition and changes in metabolism (Grant & Rees, 1957). With the development of improved methods for isolating liver nuclei free from contaminants, a more detailed study has now been undertaken.

During the 6 months of feeding, liver nuclei were isolated at various intervals from 1 to 22 weeks. Chemical analysis of these preparations showed quantitative changes in the nucleic acids, proteins and phospholipids. In view of these findings the incorporation of labelled metabolites into protein and ribonucleic acid *in vitro* was studied.

Histological studies of livers of rats fed on a range of hepatocarcinogens have been well documented (Firminger, 1955). The histological changes during the induction period vary according to the compound used. Thus it was of interest to know whether the biochemical changes observed in the nuclei after the feeding of thioacetamide are due to the carcinogenic process or to the specific substance fed. For this reason similar studies were undertaken by using the hepatocarcinogen *p*-dimethylaminoazobenzene (butter yellow).

MATERIALS AND METHODS

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

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

For incorporation of [6-¹⁴C]orotic acid and [8-¹⁴C]adenine into ribonucleic acid (RNA) the system used was: sodium phosphate buffer, pH 7.4, 20 mM; glucose, 12 mM; NaCl, 10 mM; 1 μ C of [¹⁴C]adenine or [¹⁴C]orotic acid; 1.5 ml. of nuclear suspension in 0.25 M-sucrose; water to 2.5 ml. The temperature was 30°. The reaction was stopped by the addition of 2.0 ml. of ice-cold 10% (w/v) HClO₄. The precipitate was washed four times with 2.0 ml. of ice-cold 2% (w/v) HClO₄, twice with ethanol and twice with ether.

For incorporation of [2-¹⁴C]glycine into nuclear protein the system used was: sodium phosphate buffer, pH 7.4, 20 mM; NaCl, 20 mM; [¹⁴C]glycine, 1 μ C; 1.5 ml. of nuclear suspension in 0.25 M-sucrose; water to 2.5 ml. The temperature was 38°. 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) of 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).

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

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

Medical Research Council 41 B meal (Bruce & Parkes, 1946) fed *ad lib*. Thioacetamide: 330 mg. dissolved in 20 ml. of ethanol was mixed with 1 kg. of meal. *p*-Dimethylaminoazobenzene: 600 mg. suspended in 20 ml. of ethanol was mixed with 1 kg. of meal. For the controls, 20 ml. of ethanol was mixed with 1 kg. of meal. Water was given *ad lib*.

RESULTS

Histological changes

Thioacetamide. The histological changes in the livers of animals fed with thioacetamide have been described by Gupta (1956). In our experiments the same sequence of events was observed. For the purposes of this investigation these changes have been divided into three arbitrary divisions. The first stage (1–7 weeks) shows enlargement of the parenchymal cell nuclei with an enlarged solitary nucleolus ('starry' nuclei; Gupta, 1956). Also there was some cell death with pycnotic nuclei. Towards the end of this period very slight bile-duct-cell proliferation could be observed. In the second stage (8–15 weeks) there is progressive bile-duct-cell proliferation, and towards the end cirrhosis develops. The final stage (17–22 weeks) shows similar changes, but with the onset of bile-duct-cell tumours (cholangiocarcinoma) and regeneration nodules.

***p*-Dimethylaminoazobenzene.** The histology of the livers of rats fed with *p*-dimethylaminoazobenzene has been described by Orr (1940). Similar changes were obtained in our experiments. Again three arbitrary divisions have been made covering the 22 weeks of treatment. The first stage (1–4 weeks) shows little histological change apart from a progressive inflammatory response. At 3 weeks, however, three out of four animals showed an apparent increased density of parenchymal cells in the region of the portal vessels. The second stage (5–9 weeks) shows progressive bile-duct-cell proliferation and towards the end moderate cirrhosis is present. A very striking change in this stage is the presence of large variations in nuclear size with many nuclei reaching diameters four times that of normal nuclei. The final stage (10–22 weeks) is dominated by large areas of cellular regeneration showing several mitotic figures and islands of densely packed cells.

Chemical composition

Thioacetamide. During the course of feeding thioacetamide to rats, treated and control animals were killed at 3- to 4-week intervals. Nuclei were isolated from their livers and subjected to chemical analysis. Table 1 shows the results of determining the numbers of nuclei and their nitrogen content. The nitrogen content of the nuclear fraction increases by over 50% within 2 weeks of feeding.

Since there is no rise in the nuclear numbers during the 21-week period, there is a considerable increase in the nitrogen content per nucleus. Determinations of nucleic acid phosphorus of nuclear preparations were made (Table 2). There was a rapid rise in the RNA P and a rapid but smaller rise in the DNA P expressed in terms of liver weight and per nucleus. In relation to nuclear nitrogen, the RNA P was constant and there was a slight decrease in DNA P. Similarly, phospholipid P increased per nucleus, the ratio to nuclear nitrogen remaining constant (Table 3). The results given in Tables 1–3 were obtained from the same group of animals.

***p*-Dimethylaminoazobenzene.** Similar experiments were carried out on rats fed on *p*-dimethylaminoazobenzene. At 2 weeks there was a rise in nuclear numbers which reached a maximum at 3 weeks. After this, nuclear numbers declined, and in the later stages of the experiment (14 weeks onwards) fell slightly below the levels of the controls (Table 4). A corresponding increase in nuclear nitrogen occurred as expressed in terms of liver weight, with a maximum at 3 weeks, followed by a gradual return to normal values. That the changes in nuclear numbers and nuclear nitrogen are not proportional can be seen by reference to the results of the nitrogen content per nucleus. A rise occurred in the nucleic acid content of the nuclear preparations with maximum values at 5 weeks (Table 5). The DNA values remained above those of the controls over the whole 22-week period, whereas the RNA content returned to the control level in the last 5 weeks. The nucleic acid content per nucleus, however, at 2–3 weeks is below the control

Table 1. *Nuclear numbers and total nuclear nitrogen from the livers of rats fed on thioacetamide*

Weeks of feeding	Numbers of nuclei/ μ g. wet wt. of liver	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
2	157	2.78	17.8
	126	2.30	18.3
6	155	2.75	17.8
	137	2.32	16.9
10	113	1.97	17.5
	133	2.75	20.7
13	185	2.97	16.2
	135	2.20	16.3
17	183	2.88	15.7
	152	2.66	17.7
21	146	3.22	21.8
	107	2.26	21.2

Table 2. *Nucleic acid phosphorus in liver nuclei from rats fed on thioacetamide*

The control values are the means of 17 animals killed at intervals during the 21-week period $\pm$ S.D. The other values are for individual, treated animals.

Weeks of feeding	$\mu\text{g. of phosphorus/g. wet wt. of liver}$		$\mu\text{g. of phosphorus/mg. of total nuclear nitrogen}$		$\mu\text{g. of phosphorus/nucleus}$	
	DNA	RNA	DNA	RNA	DNA	RNA
Control	115 $\pm$ 5.9	69.0 $\pm$ 2.5	72.4 $\pm$ 2.9	42.3 $\pm$ 1.4	0.99 $\pm$ 0.05	0.60 $\pm$ 0.04
2	140 141	117 107	51 61	42 46	0.87 1.09	0.73 0.79
6	147 145	129 113	53 63	47 49	0.95 1.06	0.84 0.82
10	135 151	93 129	69 55	47 47	1.19 1.13	0.82 0.97
13	151 140	130 104	51 64	44 47	0.82 1.03	0.70 0.77
17	141 131	118 105	49 49	39 39	0.77 0.86	0.64 0.69
21	138 123	147 126	43 54	46 56	0.93 1.15	1.00 1.18

Table 3. *Phospholipid phosphorus in liver nuclei from rats fed on thioacetamide*

The control values are the means of 16 animals killed at intervals during the 21-week period $\pm$ S.E. The other values are for individual, treated animals.

Weeks of feeding	Phosphorus		
	$(\mu\text{g./g. wet wt. of liver})$	$(\mu\text{g./mg. of total nuclear nitrogen})$	$(\mu\text{g./nucleus})$
Control	60.5 $\pm$ 3.6	38.1 $\pm$ 1.0	0.53 $\pm$ 0.04
2	116 125	42 54	0.72 1.00
6	125 130	45 56	0.81 0.95
10	84 109	43 40	0.74 0.82
13	132 78	44 35	0.72 0.58
17	129 82	45 31	0.70 0.54
21	165 110	52 49	1.12 1.02

levels because of the large rise in nuclear number at this period. The phospholipid analysis of the nuclear preparations presents a similar picture to that of the nucleic acids (Table 6). The results given in Tables 4–6 were obtained from the same group of animals.

Incorporation experiments

Thioacetamide. It has been shown that isolated rat-liver nuclei will incorporate [^{14}C]glycine into the nuclear protein and [^{14}C]orotic acid and [^{14}C]adenine into the nuclear RNA (Rees & Rowland, 1961). These systems have been applied to the

precancerous liver nuclei. Control and treated animals were killed at intervals during the course of feeding. The results of the incorporation experiments *in vitro* have been expressed as a percentage decrease or increase in the values for the treated animals over their corresponding controls. The incorporation of [^{14}C]glycine showed no significant change. Fig. 1 shows the percentage change in the incorporation of [^{14}C]orotic acid and [^{14}C]adenine

Table 4. *Nuclear numbers and total nuclear nitrogen from the livers of rats fed on p-dimethylaminoazobenzene*

The control values are the means of 18 animals killed at intervals during the 22-week period $\pm$ S.E. The other values are for individual, treated animals.

Weeks of feeding	Numbers of nuclei/ $\mu\text{g. wet wt. of liver}$	Total nuclear nitrogen	
		$(\text{mg./g. wet wt. of liver})$	$(\mu\text{g./nucleus})$
Control	116 $\pm$ 10.2	1.58 $\pm$ 0.04	13.7 $\pm$ 0.91
2	174	—	—
3	261	2.40	9.2
5	155	2.25	14.5
7	116	2.06	17.7
9	160	1.72	10.5
14	100	2.15	21.6
17	122 123	1.41 1.56	11.5 12.7
18	88 91	1.21 1.28	14.0 14.2
19	94 96	1.48 1.45	15.8 15.1
22	131 112 67	1.82 1.72 1.52	13.9 15.3 22.8

Table 5. *Nucleic acid phosphorus in liver nuclei from rats fed on p-dimethylaminoazobenzene*

The control values are the means of 17 animals killed at intervals during the 22-week period $\pm$ s.e. The other values are for individual, treated animals.

Weeks of feeding	$\mu\text{g. of phosphorus/g. wet wt. of liver}$		$\mu\text{g. of phosphorus/mg. of total nuclear nitrogen}$		$\mu\mu\text{g. of phosphorus/nucleus}$	
	DNA	RNA	DNA	RNA	DNA	RNA
Control	115 $\pm$ 5.9	69 $\pm$ 2.5	72.4 $\pm$ 2.9	42.4 $\pm$ 1.4	0.99 $\pm$ 0.05	0.60 $\pm$ 0.04
2	99	59	—	—	0.57	0.35
3	119	91	49	37	0.45	0.34
5	158	90	69	40	1.01	0.58
7	128	86	62	42	1.11	0.74
9	137	101	80	38	0.84	0.73
14	138	89	64	41	1.39	0.89
17	126	67	90	48	1.03	0.55
	147	75	94	48	1.19	0.62
18	167	72	138	60	1.93	0.84
	158	82	123	64	1.77	0.93
19	136	57	92	39	1.43	0.60
	123	62	85	43	1.28	0.65
22	153	60	84	40	1.16	0.90
	166	—	97	—	1.49	—

Table 6. *Phospholipid phosphorus in liver nuclei from rats fed on p-dimethylaminoazobenzene*

The control values are the means of 16 animals killed at intervals during the 22-week period $\pm$ s.e. The other values are for individual, treated animals.

Weeks of feeding	Phosphorus		
	$(\mu\text{g./g. wet wt. of liver})$	$(\mu\text{g./mg. of total nuclear nitrogen})$	$(\mu\mu\text{g./nucleus})$
Control	60.5 $\pm$ 3.6	38.1 $\pm$ 1.0	0.53 $\pm$ 0.04
2	54	—	0.29
3	97	40	0.36
5	72	32	0.44
7	94	46	0.81
9	94	32	0.72
14	86	40	0.87
17	41	29	0.34
	43	27	0.35
19	42	29	0.44
	61	42	0.63
22	68	38	0.52
	46	27	0.41
	39	26	0.59

over the control values. A rapid fall took place in the first 3 weeks and the level of incorporation remained depressed over the whole 21-week period.

p-Dimethylaminoazobenzene. Similar incorporation experiments were carried out *in vitro* during the course of feeding *p*-dimethylaminoazobenzene. Fig. 2 shows experiments on the incorporation of [^{14}C]glycine into the protein of liver nuclei. There was an initial rise, but from 3 weeks onwards the

Fig. 1. Incorporation *in vitro* of [^{14}C]adenine (●) and [^{14}C]orotic acid (○) into liver nuclei from rats fed on thioacetamide. Percentage deviation from controls. [Mean control values for 22 animals are: orotic acid 172 ($\pm$ 14) and adenine 148 ($\pm$ 15) counts/min. corrected to infinite thickness.]

values of the treated animals fell below those of the controls and remained down for the remainder of the experiment. In the incorporation of [^{14}C]orotic acid and [^{14}C]adenine into nuclear RNA (Fig. 3) a progressive rise took place over the first 10 weeks of feeding. After this values fell, and by 20 weeks had fallen below control levels.

DISCUSSION

Although the histological changes observed in the livers of thioacetamide-fed rats fall conveniently into the three stages described, the changes in the parenchymal cell nuclei, once developed, are

Fig. 2. Incorporation *in vitro* of [^{14}C]glycine into liver nuclei from rats fed on *p*-dimethylaminoazobenzene. Percentage deviation from controls. [Mean control value for 17 animals is 250 (± 18) counts/min. corrected to infinite thickness.]

Fig. 3. Incorporation *in vitro* of [^{14}C]adenine (●) and [^{14}C]orotic acid (○) into liver nuclei from rats fed on *p*-dimethylaminoazobenzene. Percentage deviation from controls. [Mean control values for 22 animals are: orotic acid 172 (± 14) and adenine 148 (± 15) counts/min. corrected to infinite thickness.]

maintained fairly steadily throughout the 22 weeks. Thus in the first histological stage, nuclear nitrogen, nucleic acid and phospholipid contents rise and remain high throughout the second and final stages. This is probably due to a different cause in each stage. In the first histological stage nuclear and nucleolar enlargement probably account for the high chemical content, whereas in the other stages it may be due to bile-duct-cell proliferation and regeneration nodules. The results of the incorporation experiments are more difficult to interpret. Thus glycine incorporation is not raised when nuclear protein is increasing. If the incorporation of glycine *in vitro* reflects protein synthesis *in vivo*,

two possible explanations for this discrepancy are (1) a decrease in the breakdown of protein in the nucleus, or (2) that nuclear preparations from the thioacetamide-fed rats contain a number of nuclei that do not incorporate glycine. Histological examination shows areas of pycnotic nuclei in these livers and their presence may mask an increased incorporation of glycine by the other nuclei. We have not confirmed the earlier findings of Grant & Rees (1957) of an increase in glycine incorporation into the nuclear preparations from the livers of thioacetamide-fed rats.

The incorporation of orotic acid and adenine into the RNA is reduced at a period when marked increase of RNA is taking place. This again could be due either to a decreased rate of breakdown of the RNA or to the presence of pycnotic nuclei in the preparation.

On feeding *p*-dimethylaminoazobenzene, the biochemical changes in the nucleus show a closer correlation with the histological stages. This may be due to the absence of pycnotic nuclei. The first histological stage with its increase in nuclear numbers at 3 weeks shows corresponding increases in the incorporation of glycine, orotic acid and adenine. Chemical analysis of the nuclear preparations also shows a rise in protein, nucleic acid and phospholipid. However, the rate at which nuclear numbers have increased must have outstripped the rate of increase of these substances since the amount per nucleus by 3 weeks has fallen below that of control values.

In the second histological stage the presence of giant nuclei is reflected in the attainment of maximum orotic acid and adenine incorporation into the RNA and maximum content of the chemical substances that were determined. The fall in glycine incorporation below control levels agrees closely with the previous findings of Grant & Rees (1957). Since there are no pycnotic nuclei it is possible that increase in protein content is the result of decreased breakdown.

In the final histological stage there was a return to normal values of the synthetic processes and the chemical composition, with the exception of the DNA levels. This may be correlated with the cellular regeneration that is taking place at this time. The large number of mitotic figures present may explain why the DNA content is still raised.

One of the difficulties in studying nuclei from the precancerous liver by these methods is that the nuclear preparation shows greater variation in nuclear types as the experiment proceeds. For example, when bile-duct-cell proliferation takes place, the ratio of parenchymal cell nuclei to bile-duct-cell nuclei decreases; also the size of the parenchymal cell nuclei changes. It could thus be argued that a more favourable approach is the use

of radioautographic techniques (Logan, Ficq & Errera, 1959). This permits the study of incorporation experiments in individual nuclei but does not give any information about the quantitative chemical changes in the nuclei.

It is of interest to know whether there are any common biochemical changes in the nuclei as a result of feeding the two carcinogens. However, the two types of nuclear preparations behaved differently in the incorporation of labelled precursors into nucleic acids and proteins, even though both groups of nuclei, however, showed increases in their protein, nucleic acid and phospholipid content. Thus, although a reasonable correlation is obtained between the biochemical and histological changes in the two groups, there is so far little evidence of any common pattern in the nuclear changes. It is therefore necessary to study more hepatocarcinogens and to compare them with compounds which produce non-malignant hyperplasia of liver and bile-duct cells.

SUMMARY

1. Nuclei were isolated in 0.25M-sucrose from the livers of normal rats and of rats fed with either thioacetamide or *p*-dimethylaminoazobenzene.

2. Nuclear counts, nuclear nitrogen, deoxyribonucleic acid, ribonucleic acid and phospholipids were determined on the nuclei of treated rats at intervals during the 22-week feeding period. In all

cases an increase over controls was obtained during the early weeks of feeding.

3. The incorporation *in vitro* of [^{14}C]glycine into protein, and [^{14}C]orotic acid and [^{14}C]adenine into RNA of the isolated nuclei was studied in control and treated animals over the feeding period. Orotic acid and adenine incorporation fell in thioacetamide-treated animals and rose in *p*-dimethylaminoazobenzene treatment.

4. The results are correlated with the histology of the livers of the treated rats and discussed in relation to carcinogenesis.

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Mammalian Fucosidases

1. THE SYNTHESIS OF SUBSTRATES AND INHIBITORS

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A comparative study has already been made of the occurrence of β -D-glucuronidase, β -N-acetyl-D-glucosaminidase, α -D-mannosidase and β -D-galactosidase in mammalian tissues (Conchie, Findlay & Levy, 1959*a, b*), and it was suggested that these enzymes could have a common function in the catabolism of mucosubstances. Since L-fucose (6-deoxy-L-galactose) is frequently found in compounds of this class, it was decided to examine the same tissues for L-fucosidase activity, and also

to test for D-fucosidase activity. D-Fucose has not been reported in animals, but it does occur in plants, and the enantiomorphs of fucose are related in the same sense as D-glucose is to L-glucose, being theoretically capable of interconversion by end-to-end oxidation-reduction. It was also possible that β -D-galactosidase would hydrolyse β -D-fucosides. The enzyme studies form the subject of subsequent communications. For convenience, the syntheses of the substrates are