Studies of Nuclei Separated by Zonal Centrifugation from Liver of Rats Treated with Thioacetamide

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1. The effects of the inclusion of thioacetamide in the diet on the properties of rat liver nuclei were studied both in adolescent rats, in which the parenchymal cells contain diploid nuclei, and in young adult rats, with a high proportion of tetraploid nuclei. 2. These investigations included a survey of the sedimentation properties of the nuclei, the nuclear volumes, content of DNA, RNA and protein, the incorporation *in vivo* of [³H]thymidine into DNA and [¹⁴C]orotate into RNA, and measurements of the activity of RNA polymerase and ribonuclease. These studies were conducted on nuclei fractionated by zonal centrifugation. 3. In both groups of animals, exposure to thioacetamide produced large numbers of nuclei that were abnormal in their chemical composition and enzymic activity. The changes were complex as regards both the types of nuclei that were affected and in their variation with time. 4. In adolescent rats two waves of synthesis of DNA and RNA were observed, one at 3 days and the other after 2 weeks of treatment. The first decline in the incorporations into both DNA and RNA coincided with a decrease in the pool sizes of some of the precursors. The activity of RNA polymerase was not substantially altered. A marked increase in the content of protein was observed before the first wave of synthesis. The normal progressive increase in tetraploid nuclei was prevented. 5. In young adult rats two waves of DNA synthesis were detected. Each was preceded by a large increase in the amount of protein per nucleus but was not accompanied by increased RNA synthesis. After 4 weeks of treatment, the diploid stromal nuclei appeared mainly unaffected and large numbers of tetraploid nuclei with a greatly increased quantity of protein were observed.

Since Fitzhugh & Nelson (1948) showed thioacetamide to be a weak hepatocarcinogen, the liver of rats fed or injected with thioacetamide has been studied from several points of view. Rather (1951) described an increase in the size of nucleoli and nuclei of parenchymal cells. Carnes *et al.* (1953) found a direct relationship between nuclear volume and content of DNA per nucleus in the liver of thioacetamide-treated rats. However, Laird (1953) and Thomson *et al.* (1953) failed to find an increase in the content of DNA per nucleus after administration of thioacetamide. On the other hand Laird (1953) reported a considerable increase in nuclear proteins and RNA per nucleus. Kleinfeld (1957) and Adam & Busch (1963) reported that this increase in nuclear RNA was mainly associated with nucleoli. Adam & Busch (1963), Steele & Okamura (1964) and Steele *et al.* (1964) demonstrated a marked stimulatory effect of thioacetamide on nucleolar RNA synthesis in liver cells. Villalobos *et al.* (1964a) described an increase in the synthesis of RNA *in vitro* by the liver nucleoli of thioacetamide-treated rats; they also showed (Villalobos *et al.*, 1964b) an eightfold increase in the nuclear ribonuclease activity after the administration of thioacetamide for 24–48 h. The

same group (Villalobos *et al.*, 1965) found that the increase in the ribonuclease activity was mainly associated with the nucleoli. Reddy *et al.* (1969) observed an increase of [³H]thymidine incorporation and of mitotic index after a single injection of thioacetamide. They also reported a significant rise in deoxycytidylate deaminase activity. Kizer *et al.* (1965, 1966) demonstrated an increase in AMP deaminase activity in liver of thioacetamide-treated rats.

Liver is an organ constituted of several classes of cells. They may be divided into two categories: parenchymal and stromal, the latter including the bile-duct cells, Kupfer cells, blood-vessel cells, connective-tissue cells etc. Therefore any sample of nuclei will be a complex mixture. A further complication arises because the nuclei from parenchymal cells become polyploid (for a review see Carriere, 1969).

In liver of newborn rats only diploid mononucleated cells are present. Shortly after birth binucleated cells with two diploid nuclei appear and rapidly increase in proportion. They reach considerable numbers before cells with one tetraploid nucleus have formed an important fraction of the cell population. As the cells with a tetraploid nucleus increase in number, binucleated cells with two diploid

nuclei decrease. The mononucleated tetraploid cells soon become the most abundant type of cell and the population of binucleated cells with two tetraploid nuclei increases, but never reaches a high proportion. Also it is possible to find a few mononucleated cells with octaploid nuclei.

The present paper describes the effect of administration *in vivo* of thioacetamide on the properties of liver-cell nuclei separated into different classes by use of the zonal centrifuge. Our purpose was to gain an insight into the mechanism of action of the carcinogen by investigation of the types of nuclei that it affects and to exploit the perturbation of the synthesis of nucleic acids, induced by thioacetamide, to uncover some of the factors regulating these processes. Animals of two different ages were studied: one group in which the polyploid nuclei had not appeared and the other with a considerable proportion of tetraploid nuclei. Determination of changes in pool size of nucleoside triphosphates and TTP caused by administration of thioacetamide were made to establish to what extent changes in RNA and DNA biosynthesis were attributable to alterations in the pools of precursors.

Materials and Methods

Animals

Four groups of male rats (Wistar Porton Strain), two of 50g and two of 200g body weight, were fed with pulverized food (M.R.C. 41). One group of each weight served as a control and the other received 0.033% (w/w) thioacetamide (pure A.R., Koch-Light Laboratories, Colnbrook, Bucks., U.K.) mixed with the food (1 kg of food was mixed with 330mg of thioacetamide dissolved in 20ml of ethanol).

The animals had access to the food and water *ad libitum* and were always killed at 08:00h. The rats were used in groups of two to six depending on their body weight at the time of death.

Each animal received 25 μ Ci of [6-³H]thymidine (22.8Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) and 2.5 μ Ci of [6-¹⁴C]orotic acid (60.8mCi/mmol; The Radiochemical Centre) per 100g body weight in 0.9% NaCl by intraperitoneal injection.

The rats were killed 1 h later by cervical dislocation. A small sample of liver was fixed in 10% (v/v) formalin for histological examination. Liver-cell nuclei were prepared from the remainder of the tissue and separated into different classes by zonal centrifugation as described by Johnston *et al.* (1968a). Three major fractions of nuclei were obtained: diploid, from stromal cells (2NS); diploid from parenchymal cells (2NP); tetraploid from parenchymal cells (4NP). The effluent was collected in 40-ml fractions; samples of each fraction were used for the following analyses:

(a) DNA, by the diphenylamine reaction (Burton, 1956) and RNA, by the orcinol method (Mejbaum, 1939);

(b) proteins, determined by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard (Armour Pharmaceutical Company Ltd., Eastbourne, Sussex, U.K.);

(c) nuclear volume and nuclear counts, as described by Johnston *et al.* (1968a);

(d) acid-insoluble radioactivity by the method of MacGregor & Mahler (1967). The redissolved precipitates were counted for radioactivity in 0.4% (w/v) 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen in toluene, with an Intertechnique ABAC SL 40 liquid-scintillation spectrometer.

Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9)

This was assayed in the three major fractions of nuclei obtained from normal liver of male rats (Wistar Porton strain), which weighed 200g when started on pulverized food, and used 1 week later.

The assay was done as follows: nuclei (44×10^6 – 175×10^6) were resuspended in 0.1ml of 0.32M-sucrose–3mM-Mg²⁺; 0.25ml of 0.1M-sodium cacodylate buffer, pH 6.5, was added and incubated in a shaker bath for 10min at 37°C. The reaction was initiated by the addition of 0.15ml of 0.08M-glucose 6-phosphate. After 10min the reaction was stopped by the addition of 1.5ml of ice-cold 10% silicotungstic acid. After 15min centrifugation at 2000g a sample of the supernatant was taken for phosphorus analysis, by the method of Chen *et al.* (1956).

Nucleoside triphosphate and TTP pools

For the measurement of the nucleoside triphosphate pool groups of two rats were used each time. Livers were freeze-clamped in liquid N₂ and homogenized in 5vol. of 0.25M-HClO₄. The insoluble material was washed once with the same volume of 0.25M-HClO₄. After filtration the nucleotides were absorbed on acid-washed charcoal (Norit, SX1; Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) and eluted with 10% (v/v) pyridine as described by Tsabot & Price (1959). The pyridine extract was freeze-dried and dissolved in water (0.25ml/g of liver). The nucleoside triphosphates and TTP were separated by using ion-exchange t.l.c. as described by Neuhaud *et al.* (1965); the nucleoside triphosphates were determined directly, and TTP was measured by isotopic dilution with [³H]TTP ([methyl-³H]thymidine 5-triphosphate, 16.2Ci/mmol; The Radiochemical Centre) diluted with unlabelled TTP to a specific radioactivity of 12750 d.p.m./nmol. A portion (15nmol) of [³H]TTP was applied with 0.01ml of liver extract to a t.l.c. plate; after development and

elution of the spots, the E_{260} was determined. The samples of TTP were dried, dissolved in 0.1 ml of water and counted in 10 ml of a modified Bray's scintillation liquid (Kay *et al.*, 1971) with an Inter-technique ABAC SL 40 liquid-scintillation spectrometer.

RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6)

RNA polymerase activity (and ribonuclease) was measured in the different classes of nuclei from the four groups of rats described under 'Animals'. The procedure in the purification and fractionation of the nuclei was the same as described above except that the effluent from the zonal centrifugation was collected in 10-ml fractions and pooled so as to obtain separately the 2NS, 2NP and 4NP nuclei, the nuclei in the gap between 2NP and 4NP [D1 nuclei; this symbol is also used for the right-hand side (i.e. faster-running fraction) of the 2NP peak in the adolescent rats] and the nuclei on the right-hand side of the 4NP peak (D2 nuclei). From each fraction samples were taken for measurement of RNA polymerase and ribonuclease activity; the nuclei were separated at 2000g for 15 min.

The RNA polymerase assay had been found to be linear with number of nuclei for each zonal fraction (Johnston *et al.*, 1968b). The conditions used were found to be optimum and it is believed that both types of RNA polymerase (A and B) were active. With the use of α -amanitin it is possible to discriminate between the two polymerases. The RNA polymerase assay was performed in a total volume of 0.5 ml containing: 11×10^6 – 110×10^6 nuclei in 0.1 ml of 0.32 M-sucrose–3 mM-Mg²⁺; 50 μ mol of Tris–HCl, pH 8.3; 2 μ mol of Mn²⁺; 6 μ mol of NaF; 50 μ l of a saturated solution of (NH₄)₂SO₄, pH 8.3; 0.5 μ mol of dithiothreitol and 1.3 μ g of α -amanitin where indicated. This mixture was incubated with shaking at 37°C for 10 min and the reaction initiated by adding 0.5 μ mol each of ATP, CTP, GTP and UTP and 1 μ Ci of [³H]UTP/[³H]uridine 5'-triphosphate, ammonium salt; 15 Ci/mmol; The Radiochemical Centre). The reaction followed linear kinetics for 30 min and was then stopped by the addition of 0.5 ml ice-cold 0.02 M-sodium pyrophosphate in 10% (w/v) trichloroacetic acid. After several washes with 5% trichloroacetic acid and ethanol–chloroform (2:1, v/v) and digestion with concentrated formic acid, the acid-insoluble material was counted for radioactivity in a modified Bray's scintillation liquid (Kay *et al.*, 1971).

Ribonuclease

The ribonuclease assay was performed by a modification of the method of Villalobos *et al.* (1964b). Some 10^7 – 10^8 nuclei were resuspended in 0.4 ml of water; 0.4 ml of 0.1 M-Tris–HCl, pH 8.2, and 0.1 ml of

bovine serum albumin (10 mg/ml) were added. After thorough mixing and incubation with shaking at 37°C for 10 min, the reaction was initiated by adding 0.1 ml of a 10 mg/ml solution of highly purified yeast RNA (Calbiochem, London W.1, U.K.). After 30 min the reaction was stopped by adding 0.5 ml of ice-cold 2.1 M-HClO₄. The precipitate was removed by centrifugation and the E_{260} was measured. The blank consisted of a similar incubation mixture in which the reaction was stopped immediately after the addition of the RNA.

All the chemicals used were analytical grade.

Results and Discussion

As shown in Fig. 1 the normal increase in body weight with age is greatly decreased in those animals exposed to thioacetamide. The effect was approximately the same in both groups of treated rats, which showed an increase of only 25–30% of that in controls. It could be argued that the inclusion of the carcinogen encourages the rats to refuse the food. However, the animals were seen to take the treated diet. Further, the alterations that we have observed in the size and chemical composition of the nuclei argue against any gross deficiency of the diet of the treated animals. Ely & Ross (1951) reported a 35% decrease in nuclear volume of liver nuclei after a protein-free diet and 13.6% decrease on a 12% casein diet. We observed an increase in the nuclear volume and the nuclear content of protein as the treatment was prolonged. There were also alterations in the nucleoli, which increased in volume and took up a more central position in the nucleus. This has been reported after parenteral administration of thioacetamide by Kleinfeld (1957) and others.

In the preparation of nuclei the yield of DNA was 0.25–0.35 mg/g of liver and it was not influenced by age or the treatment with thioacetamide. The number of nuclei per g of liver, recovered in the control rats, fell from 70×10^6 in 50 g animals to 38×10^6 when they reached 300 g body weight. The decrease in the numbers of nuclei recovered per g wet weight of liver as rats increased in age, despite the constancy in the amount of DNA/g of liver, is to be expected from the increase in polyploidy of the parenchymal nuclei. The tetraploid (4N) nuclei increase in proportion to the diploid (2N) nuclei as the animals age (for a review see Carriere, 1969).

Before considering the effects of thioacetamide it is necessary to consider some of the properties of the nuclei of control animals. The chemical composition and nuclear volumes of 2N nuclei from stromal cells (2NS), 2N nuclei from parenchymal cells (2NP) and 4N nuclei from parenchymal cells (4NP) are shown in Fig. 3a' and Table 1. The nuclei of group A of the control rats correspond to the 2NS nuclei. They are smaller in size and have a lower content of RNA and

protein than the 2NP nuclei (group B in the controls). The 4NP nuclei have twice the volume and double the chemical content of the 2NP nuclei (group C in the controls). The differences in the amounts of RNA and protein in the nuclei are not attributable to variable extents of contamination with endoplasmic reticulum. Glucose 6-phosphatase activity, which may be used as a measurement of such contamination, was low and virtually the same in all types of nuclei, when expressed as activity per mg of protein (Table 2). If any microsomal fractions were present they would be concentrated in the more slowly sedimenting nuclei, i.e. the 2NS. The nuclei from stromal cells, probably because of their smaller surface area, have the lowest activity per nucleus, and the amount of enzyme per nucleus of the tetraploid parenchymal nuclei is approximately twice that of the corresponding diploid nuclei.

Because of the complexity of the changes that occur during treatment with thioacetamide the experiments with each group of rats are described separately.

Adolescent rats

This term is used for animals that weighed 50g at the start of the treatment. The zonal profiles of the nuclei isolated from this series of animals are shown in Fig. 2. The regions of the gradients that were collected to form the zones designated A, B and C in Figs. 3 and 4 are indicated in Fig. 2.

Chemical analysis and nuclear volumes. At 50g body wt. the rats do not possess detectable numbers of tetraploid nuclei. The succession of changes in the numbers of nuclei of the three main types with increasing body weight in control animals is shown in Figs. 2(a)–2(d) and 4(a)–4(d). After 3 weeks the control rats reached 100g body wt. and tetraploid nuclei started to appear. By the time the rats weighed 200g the number of tetraploid nuclei was comparable with that of the diploid nuclei. There were only minor changes in the next 2 weeks, during which the animals increased in weight to nearly 300g (Figs. 9a and 9b).

The first change seen by phase-contrast microscopy, in the isolated nuclei, from treated animals, was an alteration in the nuclear membrane with the

appearance of a bubble or bleb. This was seen after 1 day of treatment. The alteration was also observed in the histological sample. Later the nuclei started to increase in volume, as shown in Fig. 3(f). This was

Fig. 1. Change in body weight with age of normal animals and animals treated with thioacetamide

Four groups of male rats, two of 50g and two of 200g body weight, were fed with a pulverized food (M.R.C. 41). One group of each weight served as control and the other received 0.033% (w/w) thioacetamide mixed with the food. The animals were killed at various times and the change in body weight of the control (▲) and treated (●) rats is plotted against time.

Table 1. Chemical composition and nuclear volumes of main classes of rat liver nuclei

For experimental details see the Materials and Methods section. Nuclear class: 2NS stands for diploid nuclei from stromal cells; 2NP and 4NP, for diploid and tetraploid nuclei from parenchymal cells.

Nuclear class	Content (pg/nucleus)			Ratio		Volume (μm^3)
	DNA	RNA	Protein	RNA/DNA	Protein/DNA	
2NS	5 ± 0.2	0.3 ± 0.01	15 ± 2	0.06	3.0	120 ± 30
2NP	5 ± 0.2	0.6 ± 0.02	28 ± 3	0.12	5.6	200 ± 40
4NP	10 ± 0.3	1.4 ± 0.02	58 ± 5	0.14	5.8	420 ± 60

Table 2. Activity of glucose 6-phosphatase in various classes of nuclei

For experimental details see the Materials and Methods section. Each value is the mean of two determinations. The activity of the microsomal fraction was 390 μ g of P/mg of protein.

Nuclear class	Glucose 6-phosphatase activity		
	(μ g of P/10 ⁹ nuclei)	(pg of P/mm ² of nuclear surface area)	(μ g of P/mg of protein)
2NS	15.1	128	1.01
2NP	27.1	164	0.98
4NP	59.1	226	1.02

first detectable after 3 days of treatment. The increase continued with longer treatment. Nuclei throughout the full width of the zonal gradient were affected. The increase in nuclear volumes reached its highest value after 2 weeks of treatment. Subsequently the nuclei decreased slightly in size. After prolonged treatment, for more than 6 weeks, birefringent bodies were observed in a good proportion of the nuclei.

The nucleoli increased in volume at the same time as the nuclei. The number of nucleoli per nucleus did not seem to be affected by the treatment. As soon as the nucleoli started to increase in volume they began to separate from the nuclear membrane and to take up a more central position in the nucleus.

The alterations in the content of DNA, RNA and protein per nucleus after the treatment with thioacetamide are shown in Figs. 3(e)–3(k). The first change was a decrease in the content of RNA of 50% and an increase in 35% of the protein per nucleus, after 1 day of treatment (Fig. 3e). These changes occurred throughout the whole width of the gradient.

In the initial fractions of the gradient, which correspond to the 2N nuclei from stromal cells in untreated animals, no change in the DNA content per nucleus was observed by up to 6 weeks of treatment. The concentration of DNA per nucleus in the remainder of the gradient started to increase after 3 days of treatment; at that time the changes were confined to the denser regions of the gradient. The highest values were observed after 2 weeks of treatment (Fig. 3h). Toward the outer edge of the gradient, values above the amount in 4N nuclei were obtained. However, the alterations were not restricted to this region of the gradient. The content of DNA per nucleus was approximately the same after 4 weeks of treatment, but afterwards it declined, reaching the value of the 2N nuclei throughout the gradient after 8 weeks of treatment (Fig. 3k). At 8 weeks over 50% of the nuclei of control animals were 4N. We conclude that thioacetamide altered the normal development of the rat liver nuclei and interfered with the appearance of 4NP nuclei.

Kleinfeld (1957) stated that although the content of DNA per nucleus increased after treatment with

thioacetamide there was no correlation with changes of nuclear volume. She reported an increase in ploidy, as did Koulis & Kleinfeld (1964). Our chemical determinations of DNA demonstrated an increase in DNA per nucleus as the treatment progressed in the adolescent and also in young adult rats (Figs. 3 and 8) in agreement with the work of Carnes *et al.* (1953) and Heizer (1955).

Marked changes were observed in the amounts of nuclear RNA, which are not uniform throughout the gradients. In the initial fractions corresponding in the untreated animals to the 2NS nuclei, there were only small changes up to 6 weeks of treatment. However, after 8 weeks, the RNA per nucleus increased by a factor of four in the region of the gradient containing nuclei from stromal cells (Fig. 3k). In the other parts of the gradients, after an initial decrease of 50% in the concentration of RNA per nucleus in the animals treated for 1 day (Fig. 3e), it began to increase, reaching the normal value of 2N nuclei 3 days later (Fig. 3f). It continued to increase with longer treatment. The highest values were observed in the denser region of the gradient in any given experiment. The highest concentration of RNA per nucleus was obtained after 2 weeks of treatment (Fig. 3h). After 4 weeks of treatment the values of RNA per nucleus were similar to the value of 2NP nuclei (Fig. 3k). Later it increased again to almost the same value as that obtained after 2 weeks of treatment.

The content of protein per nucleus changed in a cyclic manner with two maxima. The first occurred after 3 days of treatment (Fig. 3f), and the other at 2 weeks, when it reached its highest value (Fig. 3h). It declined later, to just above the value of 2N nuclei from parenchymal cells after 8 weeks of treatment. In the same manner as that of DNA and RNA, the content of protein increased toward the denser zones of the gradient. The large changes in the content of protein, at times when content of DNA is unaltered, could explain the increases in nuclear volume, which were also noted by Laird (1953) and by Barton *et al.* (1965).

Incorporation of [³H]thymidine and [¹⁴C]orotate and the pool sizes of the precursors of the nucleic

Fig. 2. Zonal profiles of the liver nuclei prepared from adolescent rats treated with thioacetamide as described in Fig. 1 and fractionated by zonal centrifugation

The effluent was monitored at 600nm to detect nuclei by light scattering. The charts obtained from the control (Fig. 4a-d) and treated (Fig. 4e-k) animals are shown. The regions of the gradient labelled A, B and C correspond to the same fractions shown in Figs. 3 and 4. The absence of a discrete tetraploid peak in the treated animals is apparent. The lettering of Figs. 2, 3 and 4 is as follows. Control animals: a, 1 day (50g); b, 3 weeks; c, 6 weeks; d, 8 weeks; treated animals: e, 1 day; f, 3 days; g, 1 week; h, 2 weeks; i, 4 weeks; j, 6 weeks; k, 8 weeks.

acids. The control values and changes with age are shown in the Figs. 4(a)–4(d) and are summarized in Fig. 5 and Table 3. The incorporation of [^3H]thymidine reached its highest value in the zones beyond the diploid peak in those control animals where the tetraploid peak is absent (Fig. 4a). When the tetraploid peak started to appear it was possible to observe two peaks of incorporation, one formed between the peaks of 2N and 4N nuclei and the other after the 4N nuclei peak. The latter became predominant as the animals increased in weight (Fig. 4d).

The changes after the treatment with thioacetamide are shown in Figs. 4(e)–4(k) and are summarized in Fig. 5. There were obvious changes in the zonal profiles; the nuclei instead of splitting into a diploid and a tetraploid zone, were distributed in the zonal gradient in a single broad peak with considerable numbers of nuclei in the denser region of the gradient, even in animals treated for 8 weeks. Incorporation of both [^3H]thymidine and [^{14}C]orotate occurred in the same region of the gradient, reaching the highest values towards the bottom of the gradient and not in the discrete zones found in the control animals. Incorporation of [^3H]thymidine (Fig. 5) decreased after 1 day of treatment, reaching only about 10% of the control value. However, at 3 days a maximum in the rate of incorporation of [^3H]thymidine was observed. At this time the size of the TTP pool had fallen slightly to 87% of the control value (Fig. 6a). Subsequently the size of the TTP pool fell to 30% of the original value after 1 week of treatment. At this point DNA synthesis decreased to a value considerably below that of the controls. The lack of correlation between the extent of incorporation of [^3H]thymidine and the changes of the pool of TTP indicates that the latter does not exert a dominant influence on the rate of synthesis of DNA. However, the depression of incorporation at 1 week may be attributable in part to the decrease in the size of the TTP pool. A second wave of DNA synthesis occurred with a peak between 2 and 4 weeks of treatment (Table 3). The waves of synthesis might be explained by a synchronization of DNA synthesis. Reddy *et al.* (1969) reported large increases in [^3H]thymidine incorporation and mitotic activity after a single injection of thioacetamide (50mg/kg body weight). If a large proportion of the cells became synchronized in their synthesis of DNA after ingestion of thioacetamide a biphasic incorporation of [^3H]thymidine would result. However, examination of the properties of the fractionated nuclei argues against any clear-cut synchrony.

The increase in the average content of DNA per nucleus roughly paralleled the increase in DNA synthesis as measured by incorporation of [^3H]thymidine (Figs. 4e–4k, 9c–9f). Similar effects have been reported in conditions where cell proliferation is more active than normal, for example in partial hepatectomy (Gerzeli, 1957; Grundmann & Bach, 1960), or

treatment with diethylnitrosamine (Christie & Le Page, 1961).

The mechanism by which thioacetamide increased the synthesis of DNA, as measured by the incorporation of [^3H]thymidine (Figs. 4 and 5), is unknown. During the experiment there was no marked sign of increase in the liver cell numbers as judged from the histological examination and from the recovery of nuclei. Further, the liver/body weight ratio remained unchanged despite the large increase of [^3H]thymidine incorporation. According to Rubin *et al.* (1963) there was a decrease in the half-life of total hepatic DNA when rats were fed with ethionine. Because a turnover has not been described for nuclear DNA, the half-life of the tissue DNA should represent the rate of cellular replacement. If we presume that a similar change in the half-life of liver DNA occurs after treatment with thioacetamide, the stimulation of DNA synthesis can be regarded as a response to cellular damage, even though no histological signs of necrosis are apparent.

It may be relevant that thioacetamide has the property of breaking dormancy of tubers, and it was suggested that thiol compounds may inactivate the growth-inhibitory unsaturated ketonic substances by addition reactions (Sexton, 1949). The amount of acid-soluble thiol compounds increases during the late pre-replicative period of liver regeneration in the rat (Fraser & Cater, 1967).

During the treatment with thioacetamide there was an increase in the amount of protein per nucleus (Fig. 3e) before any increase in incorporation of [^3H]thymidine (Fig. 4f). A similar change was reported during the activation of the hen erythrocyte nucleus (Harris, 1967) and might represent accumulation of protein(s) needed for the initiation of DNA synthesis and the increase in RNA synthesis in liver nuclei from thioacetamide-treated rats. The large increase in the quantity of protein per nucleus, concomitant with the increase in the [^3H]thymidine incorporation (Figs. 3f, 3h) is too great to correspond to normal accumulation of chromosomal protein during the S phase. The protein/DNA ratio was raised from 5.7 to between 8 and 10.

The incorporation of [^{14}C]orotate in the control animals paralleled the numbers of nuclei, and in the tetraploid nuclei reached approximately twice the value in the diploid peak. The synthesis of RNA (Figs. 4a–4d) was low on the left-hand side of the diploid-nuclei peak where the 2N nuclei from stromal cells are found. It has been pointed out above that in the treated animals the incorporation of [^{14}C]orotate paralleled that of [^3H]thymidine. The overall incorporation was not greatly influenced by the presence of thioacetamide in the diet (Table 3). However, there were many nuclei present that had a higher activity of incorporation than in the controls of corresponding age, for example, at 6 weeks (Figs.

4d, 4j, 5). A study of Fig. 5 shows the presence of the same type of cyclic increases and decreases in the synthesis of RNA that were observed for DNA.

The sizes of the pools of the precursors for the syn-

thesis of RNA are shown in Fig. 6(a). The size of the CTP and UTP pools did not change substantially after 3 days of treatment. In the animals treated for 1 week the size of the UTP pool decreased to 70% and

Fig. 3. Effect of thioacetamide on the content of DNA, RNA and protein in various types of liver nuclei of adolescent rats

Chemical analyses were performed and nuclear volumes were measured for nuclei obtained from different regions of the zonal gradients, designated A, B, and C. The fractions of the gradient that were collected to form these regions are shown in Fig. 2. Labelling of parts of the Figure is as for Fig. 2. Fig. 3(a) contains the mean values obtained from control animals over the complete period of the experiment. The number of 4 NP nuclei is negligible at 50 g body weight (cf. Figs. 2a and 4a). The 4 NP nuclei appear as a distinct peak at 100 g body weight (cf. Figs. 2b and 4b). The letters correspond to the zonal profiles shown in Fig. 2. ■, DNA; □, protein; ▨, RNA; ▩, nuclear volume.

the CTP to only 15% of the control value. The size of the GTP pools showed little change. However, the size of the ATP pool, after remaining constant for 3 days, decreased dramatically, by almost 50% in the rats treated for 1 week. The ADP/ATP ratio in control animals and rats treated for 3 days was approx. 1:3. During the first 3 days of treatment there was little change in the size of the total pool of precursors, but a big alteration in the rate of RNA synthesis, judged either from the maxima of incorporation (Fig. 5) or the overall value (Table 3). The pools were evidently able to sustain the elevated rate of synthesis. The fall in the incorporation of [14 C]-orotate after 1 week of treatment might be related to the sharp fall in the concentration of ATP. At this time the CTP content had also decreased dramatically. We have not determined the specific radioactivities of the precursor pools derived from orotate. Such information, even if it were available, would not afford a decisive answer to the influence of pool size on the rate of synthesis, because it is conceivable that the pool sizes and specific radioactivities may vary in individual cells over wide margins and be dependent on the extent to which the cell had succumbed to poisoning by thioacetamide. The overall pattern at 1 week, namely decreased incorporation of [14 C]-orotate and [3 H]thymidine, lowered pool sizes, and

as shown below, activity of RNA polymerase, strongly suggests cellular damage.

RNA polymerase and ribonuclease activity in different classes of nuclei. In the adolescent rats there was only a small difference in the specific activity of RNA polymerase activity between the 2NS, 2NP and D1 nuclei, with the lowest activity in the 2NS and the highest in the 2NP nuclei. The proportion of the activity insensitive to α -amanitin was higher in the nuclei from stromal cells than in the nuclei from parenchymal cells (Table 4). During the treatment with thioacetamide, there was an initial increase in RNA polymerase activity, mainly in the 2NP nuclei. However, bigger changes occurred in the α -amanitin-insensitive activity of the 2NS nuclei, which reached nearly three times the control value, a similar change taking place in the 2NP nuclei, with none in the D1 region.

After this initial rise in the RNA polymerase activity it fell, particularly in the 2NS nuclei, where it only reached 50% of the control value. However, at 3 and 7 days, even though the total polymerase activity was below the control value, the α -amanitin-insensitive proportion was higher. There was also a substantial decrease in the RNA polymerase activity in the 2NP nuclei, but without change in the amount of α -amanitin-insensitive incorporation, so that here

Fig. 4. Synthesis of RNA and DNA in liver nuclei from normal animals and rats treated with thioacetamide

Adolescent rats treated as shown in Fig. 1 received 25 μ Ci of [³H]thymidine and 2.5 μ Ci of [¹⁴C]orotic acid per 100g body weight by intraperitoneal injection 1 h before death. The purified liver nuclei were fractionated by zonal centrifugation. Portions of each fraction were used to determine the number of nuclei per 40ml fraction (■) and

the incorporation of [³H]thymidine (●) and [¹⁴C]orotate (▲) into the acid-insoluble material (expressed as d.p.m./10⁶ nuclei). To facilitate the interpretation of the diagrams the profiles of the nuclei have been hatched. Samples of the nuclei from various fractions were combined to form the regions designated A, B and C as indicated in the diagrams. Labelling of parts of the Figure is as for Fig. 2.

Fig. 5. Maximum values of incorporation of [^3H]thymidine and [^{14}C]orotate into nuclei from adolescent rats from the results summarized in Fig. 4 plotted against time

The incorporation of [^3H]thymidine in the control animals is shown as $\circ$. At 6 and 8 weeks, the upper points represent the incorporation into D2 region, and the lower points the incorporation into D1 region. The incorporation of [^3H]thymidine in the treated animals exhibits a single peak and is shown as $\bullet$. The two peaks of incorporation of [^{14}C]orotate into the nuclei from control animals are shown as Δ , the upper points being those of the 4NP nuclei and the lower from the 2NP nuclei. The incorporation of [^{14}C]orotate in nuclei from treated animals ($\blacktriangle$) showed a single maximum.

again the percentage of α -amanitin-insensitive activity rose to the control value at 7 days. The changes in the D1 nuclei were similar to those in the 2NP nuclei.

The rate of RNA synthesis was assessed by measurement of the incorporation of [^{14}C]orotate into an acid-insoluble form. The formation of the product represents a balance between synthesis and degradation. To compare the different classes of nuclei, it was necessary to determine the capacity of these classes for the breakdown as well as the synthesis of RNA. As shown in Table 5 the ribonuclease activity of 2NS nuclei was six times more than that of the 2NP nuclei in control rats. During the time of the treatment with thioacetamide there was no substan-

tial change in the ribonuclease activity in the nuclei found in the 2NP region of the zonal gradient. On the other hand the ribonuclease activity of the stromal nuclei almost doubled after 1 day of treatment and later fell to 50% below the control activity. Because of the low ribonuclease activity, the nuclei were disrupted by suspension in water before the assay. Under these conditions the enzyme was many times more active than in nuclei maintained under the conditions used for the determination of RNA polymerase.

During the treatment of the adolescent rats with thioacetamide, there were cyclic changes in the incorporation of [^{14}C]orotate. Such changes were not associated with variations of similar magnitude in the

Table 3. Total incorporation of [¹⁴C]orotate and [³H]thymidine in nuclei from control and thioacetamide-treated rats
The results were obtained from Figs. 4 and 9 by summation of the incorporations and the numbers of nuclei from the separated fractions of each of the zonal profiles.

Adolescent rats	Time	[¹⁴ C]Orotic acid (d.p.m./10 ⁶ nuclei)	[³ H]Thymidine (d.p.m./10 ⁶ nuclei)
Control	1 day	169	491
	3 weeks	77	11
	6 weeks	87	21
	8 weeks	124	21
Thioacetamide-treated	1 day	155	28
	3 days	220	252
	1 week	131	80
	2 weeks	146	1176
	4 weeks	79	1026
	6 weeks	167	467
	8 weeks	124	401
Young adult rats			
Control	3 days	461	68
	5 weeks	423	63
Thioacetamide-treated	1 day	138	39
	3 days	200	241
	2 weeks	324	19
	4 weeks	437	152

Fig. 6. Effect of thioacetamide on the pool sizes of ribonucleoside triphosphates and TTP of rat liver

Adolescent rats, treated as described in Fig. 1 for 0, 3 and 7 days are shown in Fig. 6(a), and young adult rats for 0, 1 and 2 weeks in Fig. 6(b). At the end of each of these times two animals were killed, the livers were freeze-clamped and the size of the nucleoside triphosphate and TTP pools were measured as described in the text. The results are expressed in terms of the quantities of the triphosphates per g wet weight of tissue: □, TTP; Δ, CTP; ▲, UTP; ○, GTP; ■, ATP.

activity of either RNA polymerase or ribonuclease. The activity of RNA polymerase was altered but not to the same extent. The most important change in the RNA polymerase activity (Table 4) was found in the

α-amanitin-insensitive fraction, which increased even when the total activity was decreased. The A form of RNA polymerase (α-amanitin-insensitive) is responsible for ribosomal RNA synthesis (Blatti

Table 4. *Activity of RNA polymerase in various classes of nuclei from adolescent rats*

The assay was performed as described in the text. The effluent from the zonal rotor was collected in 10ml fractions. For the control and treated animals the fractions were combined as follows: 2NS, 18–21; 2NP, 22–26; D1, 27–31 (as in Fig. 2). (–) Without α -amanitin; (+), With 1.3 μ g of α -amanitin/assay; % indicates the proportion of [3 H]-UMP incorporation insensitive to α -amanitin. D1, Nuclei on the faster running side of the 2NP nuclei.

Nuclear class	Time after initiation of treatment with thioacetamide ...	Activity (pmol of [3 H]UMP/10 ⁶ nuclei)			
		0 (control)	1 day	3 days	7 days
2NS	(–)	22.1 $\pm$ 2.3	26.6 $\pm$ 1.1	10.9 $\pm$ 0.5	9.7 $\pm$ 0.5
	(+)	4.9 $\pm$ 0.5	14.5 $\pm$ 4.0	2.7 $\pm$ 0.8	3.2 $\pm$ 0.1
	%	22	55	25	33
2NP	(–)	28.7 $\pm$ 2.9	38.0 $\pm$ 4.0	19.9 $\pm$ 1.6	20.2 $\pm$ 3.1
	(+)	3.5 $\pm$ 0.5	7.6 $\pm$ 0.9	3.8 $\pm$ 0.1	7.7 $\pm$ 1
	%	12	20	20	38
D1	(–)	32.3 $\pm$ 2.5	35.9 $\pm$ 2.1	33.9 $\pm$ 1.8	20.5 $\pm$ 0.2
	(+)	5.1 $\pm$ 0.5	5.4 $\pm$ 0.6	5.8 $\pm$ 0.1	5.9 $\pm$ 0.9
	%	16	15	17	29

Table 5. *Ribonuclease activity in various classes of nuclei from adolescent rats*

The assay was performed as described in the text. The collection of the fractions was identical with that described for Table 4.

Nuclear class	Time after initiation of treatment with thioacetamide ...	Activity (ΔE_{260} /10 ⁸ nuclei)			
		0 (control)	1 day	3 days	7 days
2NS		17.9 $\pm$ 0.6	31.6 $\pm$ 1.2	6.8 $\pm$ 1.3	6.6 $\pm$ 0.7
2NP		2.5 $\pm$ 0.1	2.4 $\pm$ 0.2	2.3 $\pm$ 0.1	3.0 $\pm$ 0.4
D1		7.0 $\pm$ 0.6	4.3 $\pm$ 0.3	7.1 $\pm$ 0.5	6.7 $\pm$ 1.1

et al., 1970). The increase in its activity after thioacetamide treatment is in agreement with the morphological changes in the nucleoli and the increase in the nucleolar RNA synthesis reported by Kleinfeld (1957) and Adam & Busch (1963).

Young adult rats

This term is used for animals that weighed 200 g at the start of the experiment. The zonal profiles from a series of experiments are shown in Fig. 7. The treatment with thioacetamide prevented the steady change in the zonal profiles seen in the controls. Instead of sharply resolved zones, broader, more diffuse bands were detected.

Chemical analysis and nuclear volume. Alterations in the appearance of the nuclei under the phase-contrast microscope identical with those seen with the adolescent rats were present in the young adult rats, but they appeared later in the older animals. The volumes of the nuclei and nucleoli began to increase after 1 week of treatment (Fig. 8) and had not declined after 4 weeks of treatment.

As the treatment progressed the slow-running peak no longer seemed to contain nuclei resembling 2NP nuclei and appeared to be composed mainly of nuclei having the characteristics, including volume and shape, of 2NS nuclei. This suggests that the 2NP nuclei are the most susceptible to thioacetamide. The faster-running peak had nuclei with volumes double those of the 4NP nuclei and with enlarged and central nucleoli (Figs. 8e and 8f).

The content of DNA per nucleus (Fig. 8) started to change at the same time as the alterations to the nuclei mentioned above. The change was not observed in the first peak, where the DNA per nucleus remained at the value of the 2N nuclei (group A). However, in the second peak the average value was nearly the same as or even above that of 4N nuclei, but the amount fluctuated between the values of 2N and 4N nuclei and between the values of 4N and 8N nuclei. As with the adolescent rats it was mainly the faster-moving nuclei that were affected.

In the animals treated for 1 week the RNA per nucleus decreased to slightly more than 40% of the control value throughout the gradient. Later it

Fig. 7. Zonal profiles of liver nuclei prepared from the young adult rats treated as described in Fig. 1 and fractionated by zonal centrifugation

The effluent was monitored at 600nm. The charts obtained from the control (Figs. 9a and 9b) and treated animals (Figs. 9c-9f) are shown. The region of the gradient labelled A, B and C correspond to the same fractions shown in Figs. 8 and 9. The lettering of Figs. 7, 8 and 9 is as follows. Control animals: *a*, 1 day (200g); *b*, 5 weeks; treated animals: *c*, 3 days; *d*, 1 week; *e*, 2 weeks; *f*, 4 weeks.

increased, the first peak having a value similar to the 2N stromal nuclei and the second nearly to the value of 4N nuclei (Fig. 8f).

The concentration of protein per nucleus increased from the beginning of the treatment in the nuclei with the DNA content of 4N nuclei, reaching a value 140% of that of controls (Figs. 8c and 8d). Later in the animals treated for 2 and 4 weeks the value of protein per nucleus is just above that of the 2NS nuclei in the first peak and substantially above the value of 4N nuclei in the second peak. This, together with the

values for content of RNA and DNA and the great enlargement of the nuclei, shows that this region of the gradient contains nuclei that are very different from the 4NP nuclei of control animals.

Incorporation of [³H]thymidine and [¹⁴C]orotate and pool sizes of the precursors of the nucleic acids. In the control animals two peaks of incorporation of [³H]thymidine were observed (Figs. 9a and 9b), one between the diploid and tetraploid peaks (D1 nuclei) and the other beyond the tetraploid peak (D2 nuclei). The D1 region contains 2N nuclei that are replicating

Fig. 8. Effect of thioacetamide on the content of DNA, RNA and protein in the various types of liver nuclei from young adult rats

Chemical analyses were performed and nuclear volumes were measured in nuclei obtained from different regions of the zonal gradients. They are designated A, B and C. The fractions of the gradient that were collected to form these regions are shown in Fig. 7. Labelling of parts of the Figure is as for Fig. 7. The mean values for the control animals are shown in Fig. 8(a'). In Figs. 8(c)–8(f) are shown the results for the treated animals (3 days; 1, 2 and 4 weeks respectively). ■, DNA; □, protein; ▨, RNA; ▩, nuclear volume.

Fig. 9. Synthesis of RNA and DNA in liver nuclei from normal animals and rats treated with thioacetamide

The young adult rats treated as described in Fig. 1 received $25\mu\text{Ci}$ of ^3H thymidine and $2.5\mu\text{Ci}$ of ^{14}C orotate per 100g body weight by intraperitoneal injection 1 h before death. The nuclei were fractionated by zonal centrifugation. Samples from each fraction were used to determine the number of nuclei per 40 ml fraction ($\blacksquare$) and the incorporation of ^3H thymidine ($\bullet$) and ^{14}C orotate ($\blacktriangle$) into the acid-insoluble material (expressed as d.p.m./ 10^6 nuclei). To facilitate the interpretation of the diagrams the profiles of the nuclei have been hatched. Samples of the nuclei from various fractions were combined to form the regions designated A, B and C as indicated in the diagrams. Labelling of parts of the Figure is as for Fig. 7.

Fig. 10. Maximum values of incorporation of $[^3\text{H}]$ -thymidine and $[^{14}\text{C}]$ orotate into the nuclei of young adult rats taken from the results shown in Fig. 9 plotted against time

In the controls the incorporation of $[^3\text{H}]$ thymidine ($\circ$) shows two peaks, the lower in the D1 region, and higher in the D2 region. There are two peaks in the treated rats ($\bullet$). The incorporation of $[^{14}\text{C}]$ orotate in the control (Δ) is almost twice as much in the 4NP as in the 2NP nuclei; in the treated rats, ($\blacktriangle$), there are two peaks of incorporation, one which remains very low (2NS nuclei) and the other which increases.

their chromatin and approaching the amount of DNA in 4N nuclei, whereas the incorporation in the D2 region represents the replication of 4N nuclei. Treatment with thioacetamide stimulated the incorporation of $[^3\text{H}]$ thymidine in waves in a manner reminiscent of the incorporation in the adolescent rats. There are two maxima, one occurring after 1

week and the other after 4 weeks (Figs. 9d and 9f). The incorporation was principally on the right-hand side of the second peak of nuclei. After a decrease at 1 day to about 40% of the original value (Fig. 9c), the incorporation of $[^3\text{H}]$ thymidine increased to six times the control value in the animals treated for 1 week (Fig. 9d). At 2 weeks of treatment there was a substantial decrease in the incorporation of $[^3\text{H}]$ -thymidine (Fig. 9e), but later, at 4 weeks of treatment, it increased again, being more than twice the normal value.

The size of the TTP pool (Fig. 6b) did not change during the period of the investigation in which the first wave of enhanced DNA synthesis occurs. The observed fluctuations in incorporation must therefore be controlled by factors other than the pool size. As with the adolescent rats, thioacetamide had a marked influence on DNA synthesis. The larger and quicker response in the adolescent rats in comparison with the young adult rats, during the treatment with thioacetamide (Figs. 5 and 10) can be related to the report of Bucher *et al.* (1961) that after partial hepatectomy DNA synthesis begins more slowly as age increases.

In the control rats the incorporation of $[^{14}\text{C}]$ -orotate was low in the 2NS nuclei, and in the 4NP nuclei was roughly double the value of the 2NP nuclei (Figs. 9a and 9b). The changes that occurred after the treatment with thioacetamide are shown in Fig. 9 and are summarized in Fig. 10 and Table 3. The incorporation of $[^{14}\text{C}]$ orotate in the first (i.e. slower-running) peak of nuclei was lower than the value for 2N nuclei from parenchymal cells and remained low for the length of the experiment (Figs. 9c–9f). However, incorporation in the second peak, which initially was decreased to a value approximately that of the 2NP nuclei of the controls, steadily increased reaching after 4 weeks the value for the 4N nuclei observed in controls.

During the first 2 weeks of treatment with thioacetamide little change was seen in the size of the UTP, CTP and GTP pools (Fig. 6b). In contrast with the adolescent rats, only a slight decline in the concentration of ATP was observed. The ratio of ADP to ATP was 1:3 in the controls, a value consistent with that of the adolescent rats.

A decrease in the content of RNA per nucleus in the young adult rats was associated with a decrease in the $[^{14}\text{C}]$ orotate incorporation in the early stage of thioacetamide treatment (Figs. 8d and 9d). As the treatment progressed the amount of RNA per nucleus in the nuclei from parenchymal cells reached a higher value than the control. However, the incorporation of $[^{14}\text{C}]$ orotate, although it recovered from the initial depression, barely reached the control value. The RNA content per nucleus also increased in the adolescent rats. It has been reported that thioacetamide decreases the amount of cytoplasmic ribosomes (Laird, 1953; Kleinfeld, 1957; Adam & Busch, 1963).

Table 6. *Activity of RNA polymerase in various classes of nuclei from young adult rats*

D1 indicates the nuclei found in the region between the 2NP nuclei and 4NP nuclei in the control animals. D2 indicates the nuclei that run faster than 4NP nuclei in the zonal gradient. The effluent from the zonal rotor was collected in 10ml fractions. For the control animals and treated animals up to 1 week of treatment the nuclear fractions were combined as follows: 2NS, 18–21; 2NP, 22–26; D1, 27–31; 4NP, 32–38; D2, 39–42. For the animals treated for 2 weeks the combination was: 2NS, 18–22; 2NP, 32–30; 4NP, 31–45 (as in Fig. 7). (–), Without α -amanitin; (+) with 1.3 μ g of α -amanitin/assay; % indicates the proportion of [3 H]UMP incorporation insensitive to α -amanitin.

Nuclear class	Time after initiation of treatment with thioacetamide ...	Activity (pmol of [3 H]UMP/ 10^6 nuclei)			
		0 (control)	1 day	1 week	2 weeks
2NS	(–)	0.73 $\pm$ 0.05	8.91 $\pm$ 0.9	8.94 $\pm$ 0.7	12.0 $\pm$ 1.0
	(+)	0	1.78 $\pm$ 0.02	1.37 $\pm$ 0.08	1.16 $\pm$ 0.2
	%	0	20	15	10
2NP	(–)	22.1 $\pm$ 2.3	24.3 $\pm$ 3.5	22.3 $\pm$ 0.8	17.8 $\pm$ 0.2
	(+)	3.7 $\pm$ 0.5	4.4 $\pm$ 0.4	4.0 $\pm$ 0.2	2.6 $\pm$ 0.6
	%	16	18	18	15
D1	(–)	42.2 $\pm$ 4.0	27.2 $\pm$ 3.6	30.0 $\pm$ 1.0	—
	(+)	7.0 $\pm$ 1.5	5.4 $\pm$ 1.2	5.8 $\pm$ 0.7	—
	%	17	20	19	—
4NP	(–)	49.2 $\pm$ 3.9	65.3 $\pm$ 1.9	73.9 $\pm$ 3.1	47.0 $\pm$ 5.0
	(+)	7.4 $\pm$ 0.3	10.9 $\pm$ 2.6	11.9 $\pm$ 0.9	13.0 $\pm$ 3.0
	%	15	17	16	28
D2	(–)	52.2 $\pm$ 0.5	53.5 $\pm$ 4.7	64.7 $\pm$ 3.0	—
	(+)	9.3 $\pm$ 1.5	6.3 $\pm$ 0.8	11.8 $\pm$ 0.3	—
	%	18	12	18	—

Although this could be caused by increased breakdown in the cytoplasm, the increase in the amount of nuclear RNA suggests that one of the effects of thioacetamide may be to block the maturation and/or transport of ribosomes from nucleus to cytoplasm.

RNA polymerase and ribonuclease activity in different classes of nuclei. The nuclei from stromal cells in the control animals had a very low RNA polymerase activity, which was completely abolished by the action of α -amanitin (Table 6). The decline in the activity of RNA polymerase in the 2NS nuclei as the control animals increase in age is evident from a comparison of Tables 4 and 6. This lowering of enzymic activity is accompanied by a decreased incorporation of [14 C]orotate and a smaller content of RNA. There appears to be a progressive switching-off of RNA synthesis with increasing age of the stromal cells. This is in contrast with the nuclei from parenchymal cells, where especially in the 4NP nuclei, there was a trend towards increased RNA polymerase activity: the 4NP nuclei had double the activity of the 2NP nuclei. The regions labelled D1 and D2 have a higher RNA polymerase activity than the preceding parenchymal nuclei. In all the paren-

chymal nuclei the proportion of the activity insensitive to α -amanitin was approximately the same.

With thioacetamide treatment there was a tenfold increase in the RNA polymerase activity in the nuclei from stromal cells, with the appearance of a fraction insensitive to α -amanitin (Table 6). The activation of the nuclei from stromal cells is an important consequence of the treatment with thioacetamide. The decline in the activity with age of this class of nuclei in control animals is noted above. Thioacetamide reverses this change.

The changes in the nuclei from parenchymal cells were not uniform. In nuclei from the region of the gradient containing 2NP nuclei there was no appreciable difference from the control RNA polymerase activity, neither in the total nor in the α -amanitin-insensitive activity. The D1 nuclei show a 25% decrease in RNA polymerase activity, with the proportion of α -amanitin-insensitive activity remaining unaltered. At the beginning of the treatment there was a 40% increase in the RNA polymerase activity in the 4NP nuclei. Later, at 2 weeks the total activity returned to the control value. However, the α -amanitin-insensitive fraction of the RNA polymer-

Table 7. *Ribonuclease activity in various classes of nuclei from young adult rats*

The assay was carried out as described in the text. The combination of the fractions of the effluent from the zonal rotor was identical with that described in Table 6.

Nuclear class	Time after initiation of treatment with thioacetamide ...	Activity ($\Delta E_{260}/10^8$ nuclei)		
		0 (control)	1 week	2 weeks
2NS		15.3 $\pm$ 0.3	4.5 $\pm$ 0.4	3.5 $\pm$ 0.3
2NP		2.0 $\pm$ 0.1	1.8 $\pm$ 0.2	3.1 $\pm$ 0.2
D1		13.6 $\pm$ 0.6	—	—
4NP		1.0 $\pm$ 0.0	2.1 $\pm$ 0.3	2.5 $\pm$ 0.2
D2		7.4 $\pm$ 0.5	8.7 $\pm$ 0.2	—

ase activity had increased to twice the control value. In the region containing D2 nuclei there was a slight increase in the total RNA polymerase activity.

The results of the assays of ribonuclease activity obtained in the untreated young adult rats (Table 7) were similar to those of the adolescent rats with highest activity in the stromal nuclei. The ribonuclease activity in the region containing D2 nuclei was half the value of 2NS nuclei. The value in D1 nuclei was nearly double that in the D2 nuclei. There was a small, but consistent, increase in the ribonuclease activity in the nuclei from parenchymal cells and a decrease in the stromal nuclei caused by exposure to thioacetamide.

As in the adolescent rats, there was no clear correlation of changes in the activity of RNA polymerase and ribonuclease with the fluctuations in the incorporation of [^{14}C]orotate nor in the nuclear contents of RNA.

Conclusions

In summary, the disturbances in the morphology, sedimentation properties, chemical composition and biological activity of liver nuclei caused by thioacetamide are complex. They are influenced by the age of the animals at the start of treatment. The quicker response of the younger animals lacking 4NP nuclei, in comparison to the older animals in which 4NP nuclei are already present at the beginning of exposure to thioacetamide, may be related to the extent of polyploidy. In the young adult animals the 2NP nuclei appear to be more susceptible to the toxic effects of thioacetamide than are the 4NP nuclei. In this sense it may be argued that increase in the level of polyploidy provides a defence mechanism. Another example of the influence of age is shown in the behaviour of the 2NS nuclei, which are still active in RNA synthesis in control adolescent rats and are not much affected by thioacetamide. In contrast, in young adult rats, thioacetamide increased RNA synthesis in the hitherto virtually inactive nuclei. Many

of the changes induced by thioacetamide occur in waves. However, at any one time these changes are not uniform in all types of nuclei. A notable example of this heterogeneity was seen in the young adolescent rats after 3 days of treatment. Although the total incorporation of [^{14}C]orotate had increased by only 34%, some nuclei had an incorporation of 1700 d.p.m./ 10^6 nuclei (fraction 3, Fig. 4f) compared with a maximum of 200 d.p.m./ 10^6 nuclei (fraction 3, Fig. 4a) in controls 3 days earlier. This represents an increase of 850%.

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