Proteins from Different Classes of Liver Nuclei in Normal and Thioacetamide-Treated Rats

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1. In normal rats the amounts of each of the main types of nuclear protein, i.e. soluble proteins, histones, non-histone chromosomal proteins and residual proteins, vary within the different classes of rat liver nuclei fractionated by zonal centrifugation. 2. Heterogeneity is observed in the non-histone chromosomal proteins prepared from different classes of liver nuclei. These differences were observed by analysis of the proteins both by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and electrofocusing electrophoresis. They are most evident between the non-histone chromosomal proteins obtained from stromal and parenchymal nuclei. However, some differences are also found for the parenchymal nuclei, between the diploid parenchymal and the tetraploid parenchymal, and between them and the nuclei involved in the synthesis of DNA respectively. 3. Drastic alterations in the nuclear proteins are found after the administration of thioacetamide. The changes observed are complex and not uniform. They vary with the age of the animal and the type of nucleus. In general an increase in the soluble proteins and non-histone chromosomal proteins and a decrease in the residual proteins is observed. There is a decrease in the specific radioactivity of soluble and residual proteins. 4. Electrophoretic analysis of the non-histone chromosomal proteins showed that specific changes occurred after administration of thioacetamide, which are different in adolescent and young adult rats.

In recent years a great deal of attention has been given to studies of the nuclear proteins because of their possible role as gene regulators [for review see Hnilica (1967) and Stellwagen & Cole (1969)]. The experiments reported in the present paper were undertaken because of the important changes observed in the liver nuclear proteins during the treatment of rats with thioacetamide, as described by Gonzalez-Mujica & Mathias (1973).

The response of the nuclei to the carcinogen is heterogeneous, varying not only with the age of the animal, but also depending on the type of nucleus. Hence the use of zonal centrifugation to separate the different classes of rat liver nuclei is necessary.

The gap in information about the nuclear proteins of the different classes of normal rat liver nuclei had to be filled before any investigation of the effect of thioacetamide could be attempted. Special interest was placed on the studies of non-histone chromosomal proteins in view of their importance as gene regulators (Hnilica, 1967; Stellwagen & Cole, 1969; MacGillivray *et al.*, 1972) and the general finding of their increased amount during carcinogenesis (Steele & Busch, 1963; Sporn & Dingman, 1966; Smith & Mora, 1971).

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Materials and Methods

Biological materials

Animals. Three groups of male Wistar rats (Porton strain) were used, one of 150 g (controls), one of 200 g (young adult treated rats) and the other of 50 g (adolescent treated rats). Treated animals received, for 2 weeks, 0.033% thioacetamide (Pure A.R.; Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) mixed with pulverized food (M.R.C. no. 41) as previously described (Gonzalez-Mujica & Mathias, 1973).

The animals received 50 μ Ci of 14 C-labelled protein hydrolysate (45 mCi/mg-atom of C; The Radiochemical Centre, Amersham, Bucks., U.K.) per 100 g body wt. in 1 ml of 0.9% NaCl, by intraperitoneal injection. They were killed 1 h later by cervical dislocation, and liver nuclei were prepared and fractionated by zonal centrifugation as described by Johnston *et al.* (1968a).

Nuclear fractions. The effluent from the zonal rotor was collected in 10 ml fractions, which were pooled in such a way as to obtain five major fractions for the control rats: diploid stromal nuclei (2NS; fractions 19–23); diploid parenchymal nuclei (2NP; fractions 24–28); diploid nuclei engaged in DNA synthesis (D1; fractions 29–33); tetraploid parenchymal nuclei (4NP; fractions 34–41); tetraploid nuclei engaged in DNA synthesis (D2; fractions 42–46). The treatment

with thioacetamide causes the appearance of many abnormal nuclei, which do not sediment in the same pattern as those from untreated rats and give rise to distorted zonal profiles (Gonzalez-Mujica & Mathias, 1973). Hence the nuclei from treated rats were collected in two main fractions. With the nuclei from adolescent rats, there were slower-running nuclei (A, fractions 12–21) and faster-running nuclei (B, fractions 23–31); from the young adult rats, they were slower-running nuclei (A, fractions 17–25) and faster-running nuclei (B, fractions 26–45).

From each nuclear fraction samples were taken for measurement of numbers of nuclei (Johnston *et al.*, 1968a) and of protein (Lowry *et al.*, 1951). The remaining nuclei were collected by centrifugation at 2000g for 15 min at 2–4°C, and the nuclear proteins were fractionated as shown in Scheme 1. Chromatin was prepared from each nuclear class by a modification of the method described by Elgin & Bonner (1970). The nuclei were extracted twice with 10mM-Tris-HCl (pH 8.0)–5mM-disodium EDTA–0.14M-NaCl (approx. 1 ml of solution per 0.5 mg of nuclear protein) and centrifuged at 200g for 15 min at 2–4°C. The crude chromatin formed a pellet and was re-suspended in 13 ml of 1.7M-sucrose in 10mM-Tris-HCl (pH 8.0)–5mM-disodium EDTA–0.14M-NaCl and centrifuged at 50000g for 2½–3 h at 4°C. The combined supernatants were called soluble proteins.

The purified chromatin was homogenized in a

suitable volume of 8M-urea (freshly deionized by the use of A.R. Amberlite monobase resin M.B.3; BDH Ltd., Poole, Dorset, U.K.) in 50mM-sodium phosphate buffer, pH 7.2, and 1.4mM-β-mercaptoethanol, by using a Polytron (Scientific Laboratory Equipment, Northern Media Supply Ltd., Hull, U.K.), for 10 s at setting no. 1. The suspension was centrifuged at 2000g for 15 min at 2–4°C and the pellet was extracted once more. The combined supernatants were called non-histone chromosomal proteins.

The insoluble material remaining after the extraction of the non-histone chromosomal proteins was suspended in ice-cold 0.2M-H₂SO₄ for 30 min with occasional agitation. It was centrifuged at 2000g for 15 min at 2–4°C. The supernatant constituted the histone fraction (Elgin & Bonner, 1970). The pellet was called residual proteins and dissolved in 1M-NaOH. Proteins were determined by the method of Lowry *et al.* (1951) by using bovine serum albumin (Armour Pharmaceutical Laboratory Co. Ltd., Eastbourne, Sussex, U.K.) and calf thymus histone [Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, U.K.] as standards.

Gel electrophoresis of non-histone chromosomal proteins

Sodium dodecyl sulphate gel electrophoresis. Gels of 100g of acrylamide/l and 2g of methylenebisacryl-

Scheme 1. *Fractionation of nuclear proteins*

For explanation see the text.

amide/l (both BDH Ltd.; recrystallized as described by Loening, 1967) were prepared in 0.1M-sodium phosphate buffer, pH7.2, containing 1g of sodium dodecyl sulphate (specially purified BDH)/l and 100ml of glycerol/l. This solution was filtered through Whatman no. 1 paper and degassed. Sufficient volume of a solution of sodium persulphate (100g/l) was added to give a final concentration of 1g/l. The solution was adjusted to 1ml of *NNN'*-tetramethylethylenediamine (TEMED; Eastman-Kodak Ltd., Kirby, Liverpool, U.K.)/l. Perspex tubes (0.5cm×6cm) were filled with this mixture and allowed to set for 30min after being overlaid with water. The gels were run at 5mA/gel for 30min with 0.1M-sodium phosphate buffer, pH7.2, containing 1g of sodium dodecyl sulphate/l as electrode buffer.

The non-histone chromosomal proteins prepared as described above were dissolved in 10g of sodium dodecyl sulphate/l and 10ml of β -mercaptoethanol/l, heated at 90°C for 5min and dialysed overnight at room temperature, against 10mM-sodium phosphate buffer, pH7.2, containing 1g of sodium dodecyl sulphate/l, 1ml of β -mercaptoethanol/l and 100ml of glycerol/l. Non-histone chromosomal proteins (150–230 μ g in 0.2ml) were mixed with 20 μ l of Bromophenol Blue soln. (5g/l) and carefully loaded on the top of a gel. Electrophoresis was carried out at 5mA/tube for 11h. During that time the Bromophenol Blue had run 6cm. A set of gels were stained overnight in Coomassie Brilliant Blue (BDH) (1g/l) in ethanol–acetic acid–water (9:2:9, by vol.) and destained in ethanol–acetic acid–water (5:2:13, by vol.). After the background colour was considerably decreased the gels were kept in 10% (w/v) acetic acid and then scanned by using a u.v. scanner (Joyce, Loebl and Co. Ltd., Gateshead, U.K.). Another set of gels was frozen on a block of solid CO₂ and sliced at 1mm intervals. Each slice was placed in a scintillation vial, dried at 120°C for 30min, and digested (Goodman & Matzura, 1971) in 0.25ml of H₂O₂ (300g/l)–aq. NH₃ (sp.gr. 0.88) (99:1, v/v; prepared in the cold shortly before use) by incubation at 50°C overnight in a capped vial. After cooling to room temperature, the digested slices were counted for radioactivity in 10ml of a modified Bray's scintillation liquid (Kay *et al.*, 1971).

The following proteins were used as standards for determination of molecular weight: bovine serum albumin (Armour Pharmaceutical Laboratories), aldolase and glutamate dehydrogenase (Boehringer, Mannheim, Germany) and pancreatic ribonuclease (Sigma), all dissolved in 10mM-sodium phosphate buffer, pH7.2, containing 10g of sodium dodecyl sulphate/l and 10ml of β -mercaptoethanol/l. The solution containing the standards was treated similarly to the non-histone chromosomal proteins, i.e. 0.2ml of it containing 20 μ g of each protein was mixed with 20 μ l of Bromophenol Blue soln. (5g/l),

loaded on top of a gel and run at the same time as the non-histone chromosomal proteins.

Electrofocusing electrophoresis. This was done as described by Gronow & Griffiths (1971). Non-histone chromosomal proteins (300–450 μ g in 2ml), prepared as previously described, were made 2mM with respect to *N*-ethylmaleimide (BDH, twice recrystallized). To this solution, 2ml of Cyanogum 41 (BDH) soln. (125g/l in freshly deionized 8M-urea) and 0.25ml of Ampholine (400g/l; LKB, London, U.K.), pH3–10, were added. After degassing, 0.75ml of sodium persulphate (3g/l in freshly deionized 8M-urea), previously degassed, was added. Two tubes (0.5cm×7.0cm) were filled and allowed to set for 1h. The electrophoresis was carried out by using H₃PO₄ (50g/l) in the anode chamber and diaminoethane (50ml/l) in the cathode chamber, at 150V for 5h. The pH gradient in the gels was checked by cutting the gels into slices of 2mm thickness, extraction of the slices with CO₂-free water (1ml) and measurement of the pH of the extract with a micro-electrode. A set of gels was soaked for 12h in 100vol. of trichloroacetic acid (125g/l) and stained as described above. Another set of gels was sliced and subsequently treated as described above.

Results and Discussion

Fractionation of proteins from different classes of liver nuclei

The amounts of the main groups of the nuclear proteins vary within the different classes of nuclei (Table 1). The values given in Table 1 for total protein are in agreement with those reported by Gonzalez-Mujica & Mathias (1973). The protein/DNA ratio is 3.2 for the stromal nuclei (2NS), 4.4–4.8 in the diploid and tetraploid parenchymal nuclei (2NP and 4NP respectively) and the tetraploid nuclei engaged in DNA synthesis (D2 nuclei); however, in the diploid nuclei engaged in DNA synthesis (D1) it was 3.5. This lower value in the nuclei active in DNA synthesis may have some correlation with reports of an increase in the export of nuclear proteins by tissues stimulated to increase their DNA synthesis, e.g. liver after partial hepatectomy (Majunder *et al.*, 1967), salivary gland after isoproterenol administration (Byrt, 1966) and kidney after folic acid administration (Baserga, 1968). Also it is in agreement with the proposal that DNA synthesis is triggered by the decrease of inhibitory factors, which may be proteins, during the pre-replicative period (Baserga, 1968).

The time-course of incorporation of ¹⁴C-labelled amino acids into nuclear proteins is shown in Fig. 1. The maximum incorporation is found 1h after administration of the precursor.

With the extraction procedure that we have used (Scheme 1) four different types of nuclear proteins were obtained.

Table 1. *Nuclear proteins from different classes of liver nuclei*

Proteins from different classes of rat liver nuclei were fractionated as shown in Scheme 1. The proteins were determined as described in the text. The values in parentheses are the percentages of the total protein in each class of nuclei.

Nuclei class	Amount of protein (pg/nucleus)				
	Soluble	Non-histone chromosomal	Histones	Residual	Total
2NS	3.70 (22.4)	5.26 (31.9)	5.63 (34.1)	1.93 (11.7)	16.52
2NP	5.80 (24.2)	8.21 (34.3)	5.92 (24.7)	4.02 (16.8)	23.95
D1	3.44 (19.3)	8.15 (45.7)	4.58 (25.7)	1.66 (9.3)	17.83
4NP	9.73 (23.0)	16.29 (38.5)	9.79 (23.1)	6.52 (15.9)	42.33
D2	7.46 (17.0)	16.84 (38.4)	9.94 (22.6)	9.00 (22.0)	43.24

Fig. 1. Time-course of incorporation of ^{14}C -labelled amino acids into nuclear proteins

Young adult rats were each injected intraperitoneally with $5\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\text{mCi/mg-atom of C}$) in 0.1ml of 0.9% NaCl . Pairs of animals were killed at 30, 60 and 120min. Liver nuclei were prepared and nuclear proteins fractionated as described in the text. □, Residual proteins; ■, total nuclear proteins; ○, non-histone chromosomal proteins; ▲, soluble proteins; ●, histones.

Soluble proteins. This term is used to designate the proteins soluble in 0.14M-NaCl . Table 1 shows that they represent between 17 and 24% of the total nuclear proteins, depending on the nuclear class. Their proportion was lower in the nuclei engaged in DNA synthesis (i.e. D1 and D2 nuclei). This may be correlated with the need for new ribosomes to meet the demand of the increased protein synthesis during the S phase (Baserga, 1968), because it is thought that much of the soluble protein is ribosomal in character.

The smallest value of soluble proteins (Table 1) was observed in the 2NS nuclei; the amount in the 4NP nuclei was almost twice that in the 2NP. However, the amount in the D1 nuclei is 50% of that in the 2NP, and the D2 nuclei showed a 25% decrease in comparison with 4NP nuclei. The incorporation of ^{14}C -labelled amino acids into the soluble proteins reaches a maximum 1h after administration of the precursor, when the specific radioactivity of soluble proteins is comparable with that of the non-histone chromosomal protein s (Fig. 1).

Non-histone chromosomal proteins. This term is used for chromosomal proteins solubilized by $8\text{M-urea}-50\text{mM-sodium phosphate buffer (pH 7.2)}-1.4\text{mM-}\beta\text{-mercaptoethanol}$. They represent between 32 and 46% of the total nuclear proteins (Table 1), depending on the nuclear class. The lowest proportion was observed in the 2NS nuclei. The proportion was at almost the same intermediate value in the 2NP, 4NP and D2 nuclei, and the highest proportion was found in the D1 nuclei. The absolute value of non-histone chromosomal proteins was lower in the stromal nuclei in comparison with the parenchymal nuclei, and within the latter the tetraploid nuclei (4NP and D2) have twice as much as the diploid nuclei (2NP and D1). These findings are in agreement with the general belief that non-histone chromosomal

proteins are more concentrated in active tissues and chromatins (Stein & Baserga, 1972; Dingman & Sporn, 1964; Frenster, 1965), since it has been shown that the stromal nuclei are less active in RNA synthesis than those derived from parenchymal cells (Johnston *et al.*, 1968b).

The amino acid analyses were in agreement with those earlier reported (Elgin & Bonner, 1970; Benjamin & Gellhorn, 1968; MacGillivray *et al.*, 1971).

The time-course of incorporation of ^{14}C -labelled amino acid (Fig. 1) is similar to that of the soluble proteins, with small differences in their specific radioactivity. A more comprehensive discussion of this type of nuclear protein will be given below.

Histones. This term is used for the chromosomal proteins soluble in $0.2\text{M-H}_2\text{SO}_4$ (Hnilica, 1967). They represent between 23 and 34% of the total nuclear proteins (Table 1). The highest proportion was observed in the 2NS nuclei, and roughly the same proportion occurred in the parenchymal nuclei (2NP, D1, 4NP and D2). The amount of histone per nucleus in the tetraploid nuclei (4NP and D2) was double that in the diploid nuclei (2NS, 2NP and D1) and the ratio histone/DNA was very close to 1 in all classes of nuclei.

The histones have the lowest specific radioactivity of any of the nuclear proteins, and during the time of these experiments they showed very little change (Fig. 1).

Residual proteins. This term was used for the proteins that remain insoluble after the extraction of histones. They represent between 10 and 20% of the total nuclear proteins (Table 1). Their proportion is lowest in the D1 nuclei and highest in the D2 nuclei. The amount of residual proteins in the 4NP nuclei was 1.56 times that in the 2NP nuclei.

The amino acid analysis showed the presence of hydroxyproline and a high proportion of glycine in this type of protein. In these characteristics they resemble the amino acid composition of collagen (Steele & Busch, 1963).

Possibly these proteins originate from the nuclear membrane. Further, their amounts per nucleus in the 2NS, 2NP and 4NP nuclei are in a close relation to the surface area, being roughly $0.022\text{pg}/\mu\text{m}^2$ of nuclear surface area (the nuclei being considered as spheres).

The residual proteins have the highest specific radioactivity within the nuclear proteins (Fig. 1) in the unfractionated nuclei.

This incorporation of ^{14}C -labelled amino acids into the protein fractions from different classes of liver nuclei after 1 h is shown in Table 2. The protein fraction with the highest specific radioactivity, in any given nuclear class, was that containing the residual proteins, except in the D2 nuclei, in which the soluble proteins showed a higher incorporation. The 2NS nuclei contain residual proteins with the highest specific radioactivity, followed by the D1 and 2NP nuclei, whose specific radioactivities are both well above those of the 4NP and D2 nuclei.

The soluble proteins have the second highest specific radioactivity with three maxima in the 2NS, D1 and D2 nuclei. The histones and non-histone chromosomal proteins reach the highest specific radioactivity in the D1 nuclei.

In HeLa cells the synthesis of histones occurs in concurrence with that of DNA during the S phase (Schoechetman & Perry, 1972). It is not clear to what extent histones of stromal and parenchymal cells of liver tissue turn over. It is possible that some of the incorporation observed in the histone fractions may be due to contamination of these samples with

Table 2. *Specific radioactivity of nuclear proteins of different classes of nuclei in normal rats*

Young adult rats were each injected with $50\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\mu\text{Ci}/\text{mg-atom}$ of C). Liver nuclei were prepared and fractionated as described in the text. From each nuclear class, proteins were fractionated as shown in Scheme 1. Samples of proteins were counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971).

Nuclei class	Sp. radioactivity (d.p.m./mg of protein)			
	Soluble proteins	Non-histone chromosomal proteins	Histones	Residual proteins
2NS	24432	6260	1356	26655
2NP	8957	7100	2340	16640
D1	15049	7300	3015	21650
4NP	6376	6570	2940	9850
D2	10100	5790	2535	5440

non-histone acid-soluble proteins of high specific radioactivity.

Gel electrophoresis of non-histone chromosomal proteins from different classes of nuclei

Electrophoresis in sodium dodecyl sulphate gels. In Fig. 2 the scans of the stained gels are shown. A complex pattern was observed with approximately 20 components. Their molecular weights range

between 13000 and 146000. They show similar characteristics, especially in the proteins with a molecular weight between 30000 and 80000, within the different classes of nuclei. This is to be expected because chromatin is likely to contain similar structural proteins and enzymes whatever its source. However, marked differences are observed between the non-histone chromosomal proteins from stromal and parenchymal nuclei. The former possess two components of mol.wt. approx. 13000 and 18900,

Fig. 2. Electrophoresis of non-histone chromosomal proteins in sodium dodecyl sulphate-polyacrylamide gels

Non-histone chromosomal proteins from different classes of rat liver nuclei (a, 2NS; b, 2NP; c, D1; d, 4NP; e, D2) were prepared as described in the text. Electrophoresis of non-histone chromosomal proteins in polyacrylamide gels (100g/l), containing sodium dodecyl sulphate (1g/l), was carried out. The gels were stained and destained as described in the text. After the background was considerably decreased the gels were scanned with a Joyce-Loebl u.v. scanner. The protein migration was from left to right. The arrow on the left marks the position reached by bovine serum albumin (mol.wt. 68000) and that on the right marks the position of pancreatic ribonuclease (mol.wt. 13700).

which were absent from the 2NP and 4NP nuclei and one of which was just visible in D1 and D2 nuclei. In the high-molecular-weight region (over 135000) four distinct components were observed in the non-histone chromosomal proteins prepared from

2NS nuclei (Fig. 2a). On the other hand, the parenchymal nuclei exhibit two bands (Fig. 2c), except the D1 nuclei, which have at least three components in approximately equal amounts. The proportion of the two proteins varies within the parenchymal nuclei. In

Gel slice no.

Fig. 3. Incorporation of ^{14}C -labelled amino acids into non-histone chromosomal proteins from different classes of rat liver nuclei

Young adult rats were each injected intraperitoneally with $50\ \mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\ \text{mCi/mg-atom of C}$) per $100\ \text{g}$ body wt. The animals were killed 1h later. Liver nuclei were prepared and fractionated as described in the text. Non-histone chromosomal proteins were obtained from each nuclear class (a, 2NS; b, 2NP; c, D1; d, 4NP; e, D2) and fractionated by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The gels were frozen on solid CO_2 , sliced at 1mm intervals and each slice was digested and counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971). The protein migration was from left to right. Results are expressed as % of the total d.p.m. loaded on the gels.

the 2NP nuclei (Fig. 2b) the protein with the higher molecular weight is in excess. On the other hand, in the 4NP and D2 nuclei (Figs. 2d and 2e) this protein is present in smaller proportion. In addition, differences in the non-histone chromosomal proteins with a molecular-weight range 86 000–135 000 prepared from stromal (Fig. 2a) and parenchymal nuclei (Figs. 2b–2e) were observed.

The incorporation of ^{14}C -labelled amino acid into the non-histone chromosomal proteins, separated by sodium dodecyl sulphate gel electrophoresis, is shown in Fig. 3. In general the patterns of incorporation agree with the pattern of the stained bands (Fig. 2). In the parenchymal nuclei (Figs. 3b–3e) the non-histone chromosomal proteins of high molecular weight contain a large proportion of the total radioactivity, amounting to over 20% for the diploid nuclei (Figs. 4b and 4c) and 40% for the tetraploid nuclei (Figs.

4d and 4e). However, in the stromal nuclei (Fig. 4a) they contain less than 10%. On the other hand, a protein of mol.wt. approx. 30 000 seems to be peculiar to the 2NS nuclei, because it is absent in the parenchymal nuclei. The D1 nuclei (Fig. 4c) have a protein of mol.wt. approx. 50 000, which contains nearly 15% of the total radioactivity; this band contains a smaller proportion of the radioactivity in the other parenchymal nuclei.

Electrofocusing electrophoresis. In general the scans of the stained gel of non-histone chromosomal proteins separated by electrofocusing electrophoresis from different classes of liver nuclei were very similar (Fig. 4). The bulk of the proteins focus between pH 6.7 and 8.9, with some small components that focus at a more acidic pH (6.5). The component from the diploid nuclei, i.e. 2NS, 2NP and D1, and in 4NP nuclei (Figs. 4a–4d), that stains most strongly focuses

Fig. 4. Electrofocusing electrophoresis of non-histone chromosomal proteins

Non-histone chromosomal proteins from different classes of rat liver nuclei (a, 2NS; b, 2NP; c, D1; d, 4NP; e, D2) were prepared and subjected to electrofocusing electrophoresis in polyacrylamide gels as described in the text. The gels were stained and destained as described in the Materials and Methods section. The gels were scanned with a Joyce-Loebl u.v. scanner. For all the gel scans, pH 3 is at the extreme right-hand side and pH 10 at the extreme left. The gradient of pH was shown to be linear between these two extremes.

at pH 7.9. In the D2 nuclei (Fig. 4e) there is a strongly staining band that focuses at pH 8. The 2NS nuclei (Fig. 4a) exhibit a less-complex pattern. The proteins focusing at pH 7.6 and 7.5 represent a lower proportion in comparison with that observed in the parenchymal nuclei (Figs. 4b and 4d). In the 4NP nuclei a protein focusing at pH 8 represents a large proportion of the total proteins, in comparison with that observed in the diploid nuclei, i.e. 2NS, 2NP and D1 (Figs. 4a-4c).

The non-histone chromosomal proteins from D1 nuclei (Fig. 4c) also showed a simple pattern, and the focusing at pH 7.9 constituted a considerable pro-

portion of the total protein. The components focusing at a lower pH value were decreased in proportion.

A similar behaviour was observed in the D2 nuclei. However, the main components from these nuclei focus at pH 8.

The pattern of incorporation of ^{14}C -labelled amino acid into the non-histone chromosomal proteins examined by electrofocusing electrophoresis (Fig. 5) resembles that observed in the stained gels (Fig. 4). However, some peaks of radioactivity focus at a lower pH than the bands observed in the stained gels. The non-histone chromosomal proteins obtained from parenchymal nuclei (Figs. 5b-5e) exhibit a

Gel slice no.

Fig. 5. Incorporation of ^{14}C -labelled amino acids into non-histone chromosomal proteins from different classes of rat liver nuclei

Young adult rats were each injected intraperitoneally with $50\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\text{mCi/mg-atom of C}$) per 100g body wt. The animals were killed 1h later, and liver nuclei were prepared and fractionated as described in the text. Non-histone chromosomal proteins were obtained from each nuclear class (a, 2NS; b, 2NP; c, D1; d, 4NP; e, D2) and fractionated by electrofocusing electrophoresis. The gels were frozen on solid CO_2 , sliced at 1mm intervals, and each slice was digested and counted for radioactivity by using the modified Bray's scintillation liquid (Kay *et al.*, 1971). For each gel diagram pH 3 is at the right-hand side and pH 10 at the left. Results are expressed as % of the total d.p.m. loaded on to the gels.

similar pattern but with a few differences. The non-histone chromosomal proteins prepared from 4NP and D2 nuclei (Figs. 5*d* and 5*e*) have a strong resemblance. The protein focusing at pH 7.9 was the component that contained the largest proportion of the radioactivity. The 2NP and D1 nuclei (Figs. 5*b* and 5*c*) have similar patterns of non-histone chromosomal proteins. The 2NP nuclei possess a component, focusing at pH 9, which contains the largest proportion of the radioactivity. Also they have some proteins focusing at a low pH, 3.3–3.5. A protein focusing at pH 9.5 was observed in the non-histone chromosomal proteins prepared from the D1 nuclei. These proteins are not histones, because at pH 10 histones are positively charged and run out of the gels (Gronow & Griffiths, 1971).

The incorporation into the non-histone chromosomal proteins from the 2NS nuclei (Fig. 5*a*) showed a more complex pattern than that observed in the stained gels. There were three components, which focused between pH 5.2–5.3.

Action of thioacetamide on the nuclear proteins

A group of adolescent rats and a group of young adult rats were treated with thioacetamide for 2 weeks, after which several alterations were observed in the general characteristics of liver nuclei, and a large stimulation of the incorporation of [³H]-thymidine and [¹⁴C]orotate (Gonzalez-Mujica & Mathias, 1973).

Because during the treatment with thioacetamide the nuclear changes were not uniform in all nuclear classes, and the major changes were observed in the faster-running nuclei (Gonzalez-Mujica & Mathias, 1973), the effluent from the zonal rotor was combined

in such a way as to obtain two nuclear fractions, designated A for the slow-running nuclei and B for the faster-running nuclei. Fraction B appears to contain parenchymal nuclei that have been altered by exposure of the rats to thioacetamide. Nuclei from parenchymal cells that have been less damaged, and stromal nuclei, which undergo less change than the parenchymal nuclei, are found in fraction A.

The alterations observed in the nuclear proteins after thioacetamide treatment are described separately for the two groups of rats used.

Nuclear proteins from adolescent rats. The nuclei in fraction A (Table 3) have a total amount of protein similar to that of the 2NS nuclei (Table 1) in the normal animals. However, there was a substantial change in the proportion of the soluble proteins, which increased by a factor of nearly 2 (Table 3). On the other hand, for the histones a change in the opposite direction was observed. A decrease in the specific radioactivity (d.p.m./mg of protein) of all nuclear fractions was found (Table 3) in comparison with that observed in the 2NS nuclei in the control animals (Table 2). The greater alterations occur in the soluble and residual proteins. In the former the specific radioactivity was decreased by a factor of 6 and in the latter by a factor of 4.

The nuclei in fraction B (Table 4) have over 40% more proteins than the normal 2NP nuclei, but a smaller amount than the normal 4NP nuclei. These changes reflected an increase in all nuclear proteins except the histones and residual proteins. The largest increase was observed in the non-histone chromosomal proteins. The amount was twice that observed in the 2NP nuclei in normal rats (Table 1). The proportion that the non-histone chromosomal proteins represent of the total nuclear proteins reached a

Table 3. *Nuclear proteins from thioacetamide-treated adolescent rats*

Adolescent rats, treated with thioacetamide for 2 weeks, were injected intraperitoneally with 50 μ Ci of ¹⁴C-labelled protein hydrolysate (45 mCi/mg-atom of C) per 100g body wt. The animals were killed 1 h later. Liver nuclei were prepared and separated into two fractions, A (slower-running nuclei) and B (faster-running nuclei), as described in the text. The nuclear proteins were fractionated as shown in Scheme 1. Samples of proteins were counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971). The values in parentheses give the percentage of each type of protein of the total nuclear protein.

Nuclear fractions ...	Amount of protein (pg/nucleus)		Sp. radioactivity (d.p.m./mg of protein)	
	A	B	A	B
Protein fraction				
Soluble	7.0 (39.7)	9.0 (26.8)	3686	5197
Non-histone chromosomal	5.0 (33.4)	16.0 (46.8)	4906	5893
Histones	3.0 (16.2)	3.5 (17.7)	1149	2353
Residual	3.5 (10.7)	3.5 (8.8)	5714	8576
Total	18.5	34		

higher value than that observed in the D1 nuclei in the controls.

The specific radioactivity was decreased in a similar way to that observed in the nuclei in fraction A. However, the changes in the soluble and residual proteins were not as drastic.

These changes in the nuclear proteins are in agreement with the general finding of increase in the non-histone chromosomal proteins during chemical carcinogenesis and in certain hepatomas (Steele & Busch, 1963; Smith & Mora, 1971; Sporn & Dingman, 1966).

After 2 weeks of treatment with thioacetamide a large increase in the incorporation of [^3H]thymidine was found in the same region of the zonal gradient taken for the isolation of fraction B (Gonzalez-Mujica & Mathias, 1973). However, the behaviour of the nuclear proteins observed at this time in these nuclei from treated animals is different from that of the nuclei engaged in DNA synthesis (D1 nuclei). Therefore the increase in DNA synthesis (as estimated from the incorporation of [^3H]thymidine) is not accompanied by the normal change of the nuclear proteins. Instead there is a disturbance of the normal pattern.

The accumulation of proteins within the nucleus after administration of thioacetamide occurs concomitantly with a decrease in the incorporation of ^{14}C -labelled amino acids. If the decrease in the specific radioactivity of the nuclear proteins is not determined by changes in the pool size of amino acid, it could be explained either by a dilution of the newly synthesized proteins in the larger amount of protein already within the nucleus, or by a decrease in the rate of synthesis. In either case the accumulation of nuclear proteins within the nucleus demonstrates an imbalance in the normal nucleus-cytoplasm exchange of proteins in response to thioacetamide. The changes in the specific radioactivity of the nuclear proteins were larger than the increase in the amount of nuclear proteins, especially in soluble and residual proteins. Probably a decrease in the rate of synthesis of nuclear proteins occurs, and this may be a consequence of the alteration reported in the endoplasmic reticulum after administration of thioacetamide (Svoboda & Higginson, 1968). The increase in the amount of nuclear proteins might correspond to a decrease in the breakdown or export or both to the cytoplasm, perhaps in the form of ribosomes. A substantial increase in the soluble proteins was observed, and these are mainly

Fig. 6. Non-histone chromosomal proteins from thioacetamide-treated adolescent rats

Non-histone chromosomal proteins were prepared from the two nuclear fractions obtained from adolescent rats treated for 2 weeks with thioacetamide: slower-running nuclei (*a* and *b*) and faster-running nuclei (*c* and *d*). The non-histone chromosomal proteins were separated by the use of sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (*a* and *c*) and electrofocusing electrophoresis (*b* and *d*). The gels were stained and destained as described in the text. The gels were scanned by using a Joyce-Loebl u.v. scanner. For all the gel scans the anode was at the right-hand side. The arrow on the left marks the position reached by bovine serum albumin (mol.wt. 68000) and that on the right the position of pancreatic ribonuclease (mol.wt. 13700).

Fig. 7. Incorporation of ^{14}C -labelled amino acids into non-histone chromosomal proteins from thioacetamide-treated adolescent rats

Animals treated for 2 weeks with thioacetamide were injected intraperitoneally with $25\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\text{mCi/mg-atom of C}$) and were killed 1h later. Liver nuclei were prepared and separated into two fractions: slower-running nuclei (a and b) and faster-running nuclei (c and d). From each nuclear fraction non-histone chromosomal proteins were prepared and separated by using sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (a and c) and electrofocusing electrophoresis (b and d). The gels were sliced at 1mm intervals, and each slice was digested and counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971). For all gel curves the anode was at the right-hand side. Results are expressed as % of total d.p.m. loaded on the gels.

Table 4. Nuclear proteins from thioacetamide-treated young adult rats

Young adult rats, treated with thioacetamide for 2 weeks, were injected intraperitoneally with $50\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\text{mCi/mg-atom of C}$) per 100g body wt. The animals were killed 1h later. Liver nuclei were prepared and separated into two fractions, A (slower-running nuclei) and B (faster-running nuclei), as described in the text. The nuclear proteins were fractionated as shown in Scheme 1. Samples of proteins were counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971). The values in parentheses are percentages of the total protein in each class of nucleus.

Nuclear fraction ... Protein fraction	Amount of protein (pg/nucleus)		Sp. radioactivity (d.p.m./mg of protein)	
	A	B	A	B
Soluble	13 (50.6)	16.5 (35.3)	3223	5224
Non-histone chromosomal	7.5 (29.2)	21.0 (45.9)	7086	5998
Histones	4.0 (15.0)	6.5 (14.0)	2569	3539
Residual	1.5 (4.9)	2.5 (4.8)	6188	8556
Total	26	46.5		

composed of ribosomal proteins (Frenster *et al.*, 1960; Wang & Wang, 1962).

(a) Separation of non-histone chromosomal proteins by sodium dodecyl sulphate gel electrophoresis. The patterns of the non-histone chromosomal proteins observed in the stained gels (Figs. 6a and 6c) were very similar to each other in the two fractions of nuclei obtained from the treated adolescent rats. They closely resemble the behaviour of the non-histone chromosomal proteins prepared from the parenchymal nuclei in control animals (Figs. 2b–2e). However, differences in the relative proportion that each band represents were observed. A substantial increase in the relative amount of a component (perhaps two) of mol.wt. approx. 33 000 was observed in the non-histone chromosomal proteins prepared from both types of nuclei.

The incorporation of ^{14}C -labelled amino acid in the non-histone chromosomal proteins separated by sodium dodecyl sulphate gel electrophoresis are shown in Fig. 7. The slow-running nuclei (type A) exhibit a pattern of incorporation (Fig. 7) very similar to that observed in the parenchymal nuclei in the controls (Figs. 3b–3e). On the other hand in the type B nuclei two bands of incorporation were observed, at mol.wt. 22 000 and 51 000 respectively. They re-

present a larger proportion of the total radioactivity than the proteins in the high-molecular-weight region. The latter represent the largest proportion in the parenchymal nuclei in the controls (Figs. 3b–3e).

(b) Electrofocusing electrophoresis. The behaviour of the non-histone chromosomal proteins obtained from both types of nuclei (Figs. 6c and 6d) closely resembles that of the controls (Fig. 4). However, some components that focus at a lower pH (5.5–5.7) were present in the faster-running nuclei (fraction B) (Fig. 6c), which were not observed in the controls. The pattern of incorporation of ^{14}C -labelled amino acids in both types of nuclei (Fig. 7c) did not show the distinct peaks observed in the controls.

Nuclear proteins from young adult rats. The nuclei in fraction A (Table 4) have slightly more total protein than the 2NP in the controls (Table 1). However, the relative amounts of each class of protein have been substantially altered by the treatment with thioacetamide. The soluble proteins represent 50% of the total nuclear proteins (Table 4), which is more than double that observed in any of the nuclear classes in controls. All the other protein fractions are decreased in proportion. The residual proteins decrease to 25% in comparison with the 2NP nuclei. As with the adolescent rats the specific radioactivity of

Fig. 8. Non-histone chromosomal proteins from thioacetamide-treated young adult rats

Non-histone chromosomal proteins were prepared from the two nuclear fractions obtained from young adult rats treated for 2 weeks with thioacetamide: slower-running nuclei (a and b) and faster-running nuclei (c and d). The non-histone chromosomal proteins were separated by the use of sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (a and c) and electrofocusing electrophoresis (b and d). The gels were stained and destained as described in the text. The gels were scanned by using a Joyce-Loebl u.v. scanner. For all gel scans the anode was at the right-hand side. The arrow on the left marks the position reached by bovine serum albumin (mol.wt. 68 000) and that on the right the position of pancreatic ribonuclease (mol.wt. 13 700).

the soluble and residual proteins decreases in treated animals.

The nuclei in fraction B (Table 4) have more proteins than the 4NP nuclei in the control animals (Table 1). The amount of histones was just above the amount in the diploid nuclei in the controls. The non-histone chromosomal proteins increase by over 25% in comparison with the 4NP nuclei. Bigger changes occur in the soluble and residual proteins. The former increase by 65% and the latter decrease by a factor of 2.4 in comparison with 4NP nuclei.

The specific radioactivities of the proteins (fraction B, Table 4) are approximately the same as that observed in the 4NP nuclei in controls. All the protein fractions exhibit a higher specific radioactivity than that observed for the nuclei in fraction A, except the non-histone chromosomal proteins.

The changes found in these animals were in keeping with those observed in the adolescent rats. However, the increases in the amount of nuclear proteins were not accompanied by changes in the specific radioactivity. Therefore the accumulation of proteins in the nucleus may correspond to an increase in their synthesis.

In both groups of rats a decrease in the amount of residual proteins and in their specific radioactivities was observed.

The possible origin in the nuclear membrane of the residual proteins has been mentioned, and the alterations which occur in them may reflect damage to these nuclear structures by thioacetamide.

(a) Separation of non-histone chromosomal proteins by sodium dodecyl sulphate electrophoresis. The scans of the stained gels are shown in Figs. 8(a) and 8(c). The patterns of non-histone chromosomal proteins prepared from nuclei in fraction A (Fig. 8a) resemble those observed for the 2NS nuclei in the controls (Fig. 2a). However, the two components of lower molecular weights (18000 and 13000) were present in a larger proportion than in the 2NS nuclei.

The nuclei in fraction B (Fig. 8c) showed a protein, of mol.wt. approx. 13000, not present in the parenchymal nuclei of the controls (Figs. 3b and 3c).

The incorporation of ^{14}C -labelled amino acids into the non-histone chromosomal proteins from the nuclei in fraction A (Fig. 9a) resembles that observed for the same fraction in the adolescent rats.

The nuclei in fraction B (Fig. 9c) show a pattern of

Fig. 9. Incorporation of ^{14}C -labelled amino acids into non-histone chromosomal proteins from thioacetamide-treated young adult rats

Animals treated for 2 weeks with thioacetamide were injected intraperitoneally with $100\mu\text{Ci}$ of ^{14}C -labelled protein hydrolysate ($45\text{mCi/mg-atom of C}$) and were killed 1h later. Liver nuclei were prepared and separated into two fractions: slower-running nuclei (a and b) and faster-running nuclei (c and d). From each nuclear fraction non-histone chromosomal proteins were prepared and separated by using sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (a and c) and electrofocusing electrophoresis (b and d). The gels were sliced at 1mm intervals, and each slice was digested and counted for radioactivity in the modified Bray's scintillation liquid (Kay *et al.*, 1971). For all the gel curves the anode was at the right-hand side. Results are expressed as % of total d.p.m. loaded on the gels.

incorporation closely resembling that observed for the parenchymal nuclei in the controls.

(b) Electrofocusing electrophoresis. The scans of the stained gels showed a very similar pattern (Figs. 8b and 8c) and both resemble that obtained for the 4NP nuclei.

The incorporation of ^{14}C -labelled amino acids into the non-histone chromosomal proteins separated by electrofocusing electrophoresis of nuclei in fraction A is similar to that of the stromal nuclei.

The nuclei from fraction B (Fig. 9d) exhibit some components focusing at a lower pH than that observed in the controls (i.e. 3.2, 3.5 and 4.5). They constituted a large proportion of the total radioactivity.

Conclusions

Heterogeneity was found in the proteins from different classes of rat liver nuclei and changes were observed after thioacetamide treatment.

This work has demonstrated major differences between the non-histone chromosomal proteins of the rat liver stromal nuclei in comparison with the parenchymal nuclei, and less-pronounced differences within the various types of parenchymal nuclei in normal rats.

The parenchymal nuclei are more active in the synthesis of RNA, both *in vivo* and *in vitro* (Johnston *et al.*, 1968b; Gonzalez-Mujica & Mathias, 1973), than the stromal nuclei. The amount of histones in the 2NS nuclei is the same as that in the 2NP nuclei. The amount of non-histone chromosomal proteins in the 2NP nuclei is 60% more than in the 2NS nuclei. Although many components are similar, some proteins present in the 2NS nuclei are absent from the 2NP nuclei and vice versa. These observations are compatible with the proposal that the non-histone chromosomal proteins are involved with regulation of gene function, although they do not prove this hypothesis. The complex changes that are observed as a result of the inclusion of thioacetamide in the diet, and in particular the massive increments in the total amounts of the non-histone chromosomal proteins and of some specific components at a time when the synthesis of nucleic acids is greatly disturbed, are a further indication of the importance of this group of proteins. The complexity of the changes wrought by thioacetamide and their dependence on the age of the animals are reflected in the changes in the zonal profiles (Gonzalez-Mujica & Mathias, 1973). These experiments have high-lighted the need to characterize in greater detail the non-histone chromosomal proteins and to assign biochemical functions

to the multitude of proteins found in this class of nuclear protein.

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