A Disappearance of a 24-kDa Acid-Soluble Protein from Liver Chromatin of Normal and Starved Hens Following D-Galactosamine Administration

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Normal and starved adult chickens were injected intraperitoneally with D-galactosamine hydrochloride (0.5 g/kg body weight) and 6 h later liver chromatin acid-soluble proteins were isolated. These proteins were resolved by a two-dimensional polyacrylamide gel electrophoresis in the presence of non-ionic detergent, Triton X-100, in the first dimension and anionic detergent, sodium dodecyl sulfate, in the second dimension. Although spotting patterns of acid-soluble chromatin proteins were remarkably similar between normal and starved control birds and those receiving D-galactosamine, a disappearance of a 24-kDa protein after administration of this agent was found. Moreover, it was shown that this protein was also completely absent in the chicken erythrocyte chromatin which was known to be inactive in RNA synthesis.

It seems that the disappearance of the 24-kDa chromatin protein may be associated with inhibiting of transcription in hen liver after D-galactosamine administration and during hen erythrocyte maturation.

Introduction

Starvation is a known factor influencing the physiological properties of tissues by producing a hypometabolic environment for biochemical reactions. It has been shown [1] that morphology of liver nuclei was altered in rats subjected to a short period of food deprivation. Under this circumstances the condensed chromatin occupied much larger areas than in normal animals and the perichromatin fibrils which surrounded it were largely absent. It has been also found [2] that fluorescent spectra of chromatin and its components − histones and nonhistone proteins − isolated from starved rats were altered in comparison with those of normal ones. Moreover, a marked differences in the amount of some specific nuclear globulins with molecular weights of about 80 and 125 kDa from liver of starved rats were also observed [3, 4].

Histones play a significant role in chromatin by participating in the maintenance of chromatin structure and acting as repressors of template activity [5]. Although histones have been well characterized many years ago [6], a detailed investigations employing high resolution electrophoretic methods have revealed the existence of minor histone variants in acid extracts of chromatin [7−9]. It has been demonstrated [10, 11] that histone variants are additionally multiplied by attaching of ubiquitin − a nonhistone chromatin protein extractable with a low ionic strength solutions [12] − to the core histones, especially H2A and H2B, producing a series of minor histone-related proteins differing in their electrophoretic properties. In contrast to the whole histone bands, they have been shown to change in various tissues [13, 14], mutant cells [15], during development and differentiation [16−18] and following exposure to pharmacological agents [19].

Dilute solutions of hydrochloric, sulfuric or perchloric acids widely used for solubilizing of histones can also extract a certain number of other basic and/or neutral protein components existing in minute quantities [9, 20, 21]. Though they were found to be abundant in acid extracts of whole nuclei, their number and quantity decreased markedly in acid extracts of chromatin [7] suggesting that they were largely the nonhistone nuclear proteins readily removable from nuclei by low ionic strength salt solutions in the course of chromatin isolation. Some of them have been partially characterized. For example, proteins C23 and B23 have been isolated from nucleoli of Novikoff hepatoma cells [22] and demonstrated to be a major silver staining proteins of the nucleolar organizer [23]. A high-molecular-weight acid-soluble nuclear protein similar to protein C23 has been also prepared from mouse ascites sarcoma

cells [24]. A basic sulfuric acid-soluble protein with molecular weight comparable to that of H1 histone was obtained from calf thymus chromatin and proved to be a nonhistone protein coextracting with histones [25]. Another acid-soluble proteins, the LSNHPs (liver specific nonhistone proteins) with molecular weights of about 50 kDa have been recently described [26] to increase in chromatin of spontaneous hepatomas of ageing CBA mouse strain.

In this report D-galactosamine that is considered to be a potent inhibitor of liver transcription [27, 28] was used as a probe for seeking for differences in the gel patterns of histones and other acid-soluble chromatin proteins between normal and fasted hens. Here additional evidences are also presented which indicate that a unique acid-soluble protein with molecular weight of 24 kDa decreases not only in chromatin of normal and starved hens after administration of D-galactosamine but also in chromatin of mature chicken erythrocytes which are known [29] to be inactive in RNA synthesis. It is worth mentioning that in this laboratory a specific nonhistone protein with molecular weight of approximately 150000 was recently detected [30] which disappeared not only from chromatin of adult hens containing less RNA but also from chromatin of the new-hatched chicks treated with D-galactosamine. The above observations seem to suggest that appearance of some nuclear proteins may strongly depend on actual RNA synthesis in cells.

Materials and Methods

Animals

One-year-old Rhode Island Red hens were throughout used for experiments. Twelve hens were randomly divided into two groups of 6 birds in each. The first group was starved for 9 days and the other group was fed ad libitum a commercial mash. Three fed hens as well as three starved hens were injected intraperitoneally with 20% D-galactosamine hydrochloride (Chemapol, Czechoslovakia) in 0.15 M NaCl in the dose of 0.5 g per kg of body weight. The remaining birds were injected with 0.15 M NaCl solution only. Injections were constantly given at 8−10 o'clock a.m. and after 6 hours hens were sacrified by decapitation. A liver perfusion was performed following opening the abdomen cavity using a cold solution containing 0.15 M NaCl and 0.015 M sodium citrate.

Additionally, 6 hens of the same age were used for preparing of erythroid cells. Three of them were made anemic by giving intramuscularly a neutralized 1% phenylhydrazine-HCl solution in 0.15 M NaCl in the dose 10 mg/kg body weight for 5 consecutive days to produce reticulocytes. The control and anemic hens were killed by decapitation and blood was immediately collected into a cold solution of 0.15 M NaCl, 0.015 M sodium citrate supplemented with 0.1 mM phenylmethanesulfonyl fluoride. Erythroid cells were pelleted by centrifuging at 3000 U/min for 10 min in Janetzki K-26 centrifuge. Serum and buffy coat (containing white cells) of pellet were removed by aspiration. Cells were washed twice more with saline-sodium citrate.

Isolation of nuclei

Hen liver nuclei were isolated by a slight modification of the citric acid method described by Sippel *et al.* [31]. After excising and rinsing with saline-sodium citrate, the livers were passed through a tissue press. The disrupted tissue was homogenized in 0.025 M citric acid in a Teflon-glass homogenizer. The homogenate was first filtered through 8 layers of chirurgical gauze and then through a layer of Miracloth (Calbiochem). The filtrate was centrifuged for 10 min at $1500 \times g$. The crude nuclear pellet was resuspended in 0.025 M citric acid containing 0.03% Triton X-100 and centrifuged. After resuspending in 0.25 M sucrose and 0.01 M citric acid, the nuclei were pelleted through a cushion of 0.88 M sucrose and 0.01 M citric acid by centrifuging for 10 min at $1500 \times g$. This procedure was repeated once more. The nuclei obtained by this method were free from cytoplasmic tags judging from microscopic evaluation following staining with toluidine blue.

Both reticulocyte and erythrocyte nuclei were isolated in similar manner by a modification of previously described procedure [32]. The cells were resuspended in RSB buffer (0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.003 M MgCl$_2$) in a Teflon-glass homogenizer. An appriopriate volume of 10% Nonidet P-40 was added to a final concentration of 0.5% followed by 8 strokes of a Teflon piston. Nuclei were additionally washed twice with RSB buffer alone. After resuspending in RSB buffer containing 0.25 M sucroce, the nuclei were underlayered with 0.88 M sucrose in RSB. This procedure was repeated once more.

Isolation of histones

Histones from livers, erythrocytes and reticulocytes of hens were isolated in similar manner. Nuclei were washed twice with 0.075 M NaCl, 0.025 M disodium salt of ethylenediamine tetraacetic acid (pH 7.6) and pelleted by low-speed centrifugation. The resulted chromatin was extracted twice with 5 M urea in 0.05 M sodium phosphate buffer (pH 7.6) containing 0.02 M glycine and centrifuged for 20 min at $12\,000 \times g$ to remove a majority of nonhistone proteins. All solutions used for chromatin isolation and extraction were supplemented with 0.1 mM phenylmethanesulfonyl fluoride. The chromatin depleted of most of nonhistone proteins was extracted twice with 0.25 M HCl to obtain histones and other acid-soluble proteins. The supernatants containing acid-soluble chromatin proteins were saved and combined. After filtering through a sintered-glass funnel, the proteins were precipitated overnight with 20% trichloroacetic acid. The precipitate was washed once with acidified acetone (1 cm^3 of concentrated HCl in 250 cm^3 of acetone) and twice with acetone and dried in a cold stream of air.

Electrophoretic analysis

Protein samples were solubilized in 8 M urea, 10% 2-mercaptoethanol and 0.9 M acetic acid and after protein determination by the method of Bramhall [33], they were separated by a slight modification of a two-dimensional polyacrylamide gel electrophoresis described by Unger-Ullmann and Modak [34].

The first dimensional cylindrical gel was prepared by pouring a deaerated gel solution containing 12% acrylamide, 0.252% N,N′-methylenebisacrylamide, 0.125% N,N,N′,N′-tetramethylethylenediamine, 0.125% ammonium persulfate, 0.36% Triton X-100, 7.5 M urea and 0.9 M acetic acid into a 3×120 mm glass tubes to a height of 88 mm and leaving to polimerize for at least 3 h. Preelectrophoresis was conducted overnight at 70 V after inserting the gel tubes into the apparatus with tanks filled with 0.9 M acetic acid. The gel surfaces were overlaid with 7.5 M urea, 0.9 M acetic acid and 0.36% Triton X-100. After completion of preelectrophoresis, the gel surfaces were washed with 0.9 M acetic acid and the gel tubes and electrophoretic chambers were refilled with a fresh portion of the electrode solution. Samples containing approximately 300 µg of protein were

carefully underlaid through the electrode solution using a Hamilton syringe. Electrophoresis was conducted at 120 V until a tracking dye, a blue component of methyl green, reached the bottom of the tube.

Gels removed from the tubes were immersed in a solution of 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue and 0.01 M phosphate buffer (pH 7.6) and soaked three times for 15 min at room temperature. The soaked cylindrical gel was placed immediately on the top of a second-dimension slab gel. The running gel contained 12.5% acrylamide, 0.33% N,N′-methylenebisacrylamide, 0.025% N,N,N′,N′-tetramethylethylenediamine, 0.03% ammonium persulfate, 0.1% sodium dodecyl sulfate and 0.375 M Tris-HCl (pH 8.8). The stacking gel contained 4.5% acrylamide, 0.12% N,N′-methylenebisacrylamide, 0.1% N,N,N′,N′-tetramethylethylenediamine, 0.03% ammonium persulfate, 0.1% sodium dodecyl sulfate and 0.125 M Tris-HCl (pH 6.8). The running buffer contained 0.1% sodium dodecyl sulfate, 0.192 M glycine and 0.025 M Tris. Electrophoresis was conducted at 15 mA/slab until a tracking dye, bromophenol blue, reached 0.5 cm from the bottom of the gel.

Gels were immersed in 25% isopropanol and 10% acetic acid for 2 h and stained overnight with 0.05% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% acetic acid. The gels were destained first in 0.0035% Coomassie Brilliant Blue R-250 in 10% isopropyl alcohol and 10% acetic acid for 6 h and then in several changes of 10% acetic acid.

The molecular weights of proteins were determined according to Olson *et al.* [35] using lysozyme (mol. wt. 14300), chymotrypsinogen A (mol. wt. 25500), ovalbumin (mol. wt. 45000) and bovine serum albumin (mol. wt. 68000) as standards.

Isolation and electrophoresis of rabbit liver histones

Rabbit liver nuclei were isolated by a citric acid-sucrose method of Taylor *et al.* [36]. Nuclei were washed twice with 0.14 M NaCl, 0.05 M Tris-HCl (pH 7.5) and then twice with 0.35 M NaCl, 0.02 M Tris-HCl (pH 7.5). Histones were isolated from chromatin with 0.25 M HCl as described above. Proteins were separated by the two-dimensional gel electrophoresis exactly as described above with the exception of lower content of acrylamide (8%) and

N,N'-methylenebisacrylamide (0.17%) in the first-dimensional gel.

Determination of protein, RNA and DNA

Protein was determined by the method of Lowry *et al.* [37]. RNA was separated from DNA as described by Munro and Fleck [38]. RNA and DNA contents were assayed according to the methods of Lin and Schjeide [39] and Giles and Myers [40], respectively.

Results

Aliquots of chromatins from livers of normal and experimental hens withdrawn during their isolation were used for protein, RNA and DNA determinations. Results are presented in Table I. The content of RNA decreased by about of 46% and 35% in chromatin of starved and D-galactosamine-treated hens, respectively. Administration of the galactosamine to the starved hens did not change further
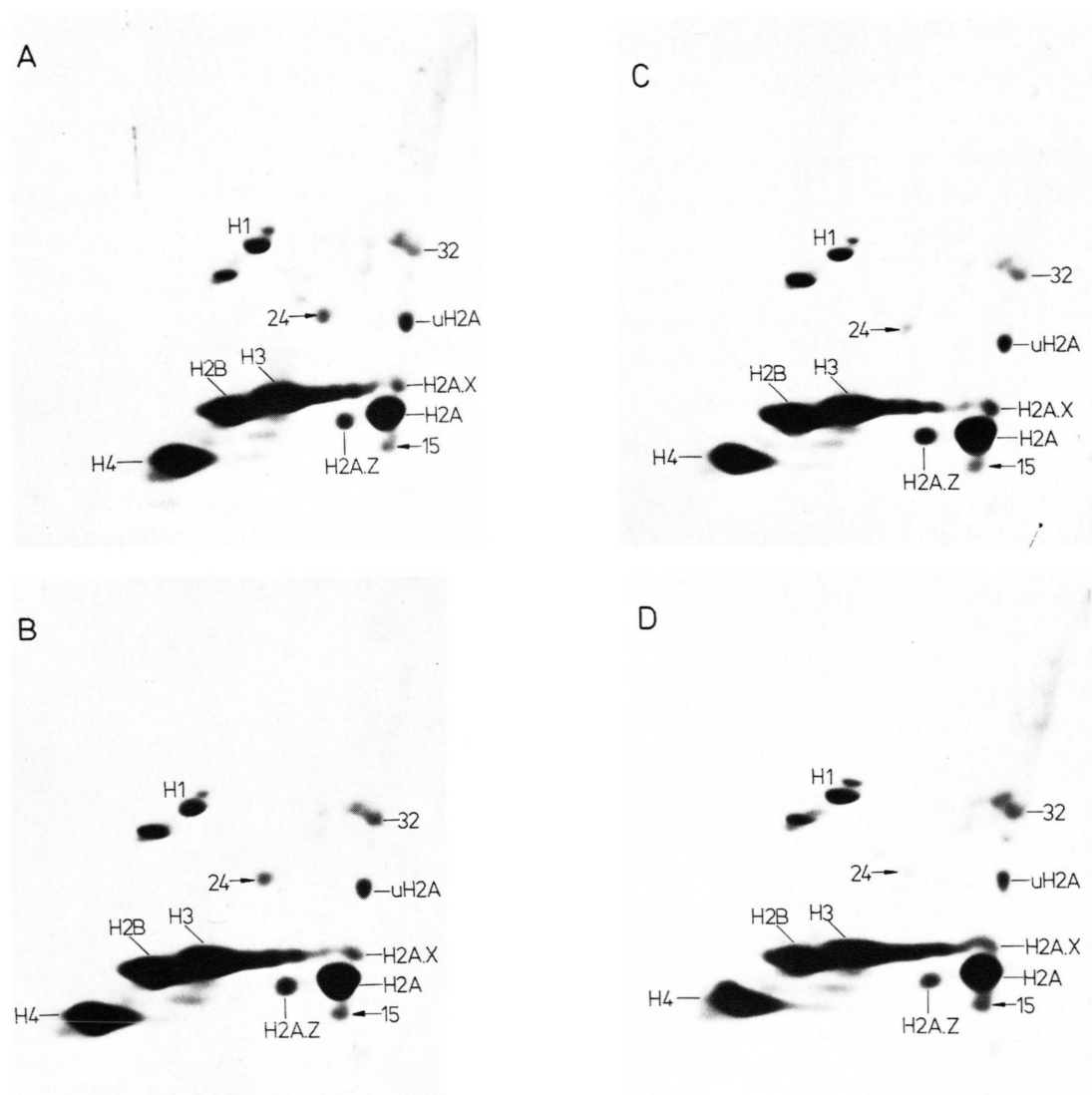

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of acid-soluble chromatin proteins from liver of normal (A), starved (B), galactosamine-treated (C) hens and starved hens injected with galactosamine (D). Approximately 300 μg of protein was separated in the first dimension in 12% acetic acid-urea polyacrylamide gel containing 0.36% Triton X-100 and in sodium dodecyl sulfate slab gel in the second dimension. Protein spots were designated according to West and Bonner [10].

Table I. The content of RNA and protein in the liver chromatin of normal and starved hens exposed to D-galactosamine. Values represent mean ± SD of 3 separate experiments.

Hens	RNA/DNA	Protein/DNA
Normal	0.097 ± 0.023	2.67 ± 0.19
Starved	0.052 ± 0.015	2.27 ± 0.22
Galactosamine-treated	0.063 ± 0.024	2.04 ± 0.23
Starved and galactosamine-treated	0.053 ± 0.028	1.91 ± 0.21

All experimental values were significantly different from control (p < 0.001, Student's t-test).

the amount of RNA in chromatin. It is apparent from Table I that diminution of RNA level was accompanied by a decrease of chromatin protein content both in starved and galactosamine-injected hens.

Acid-soluble proteins isolated from liver chromatin of control and experimental birds were resolved by a two-dimensional polyacrylamide gel electrophoresis in the presence of non-ionic detergent, Triton X-100, in the first dimension and anionic detergent, sodium dodecyl sulfate, in the second dimension (Fig. 1). This method separated hen liver H2A histone into several well visible variants (H2A.Z, H2A.X and uH2A) which were designated according to West and Bonner [10] and allowed to obtain in addition to main H3 histone spot at least three molecular species of this histone. They appeared regardless of gel concentration in the first dimension gel for they were abundant in the patterns of rabbit liver histones separated in 8% polyacrylamide gel (Fig. 3). The main histone spots and these histone variants were almost unchanged not only during starvation but also after D-galactosamine application. However, an increase of the amounts of 15- and 32-kDa proteins was observed as a result of hen starvation (Figs. 1B and D). After administration of D-galactosamine to normal hens, a sharp decrease of 24-kDa chromatin protein was detected (Fig. 1C). It almost completely disappeared from the gel pattern of acid-soluble chromatin proteins isolated from liver of starved hens receiving D-galactosamine (Fig. 1D). It seems that this protein was not only specific for hen liver chromatin since it was also abundant in rabbit liver chromatin (Fig. 3) and was as well present in much lower quantity in hen reticulocyte

chromatin (Fig. 2A). Diminution of this protein from histone pattern of galactosamine-treated hens (Fig. 1C) was accompanied by a decrease of total RNA and protein content in their liver chromatin (Table I). Food deprivation caused almost complete loss of 24-kDa protein (Fig. 1D) because starved hens had already had a lower content of RNA and protein in chromatin before D-galactosamine administration. Interestingly, the amount of uH2A protein was also somewhat lower in chromatin of starved hens receiving D-galactosamine (Fig. 1D).
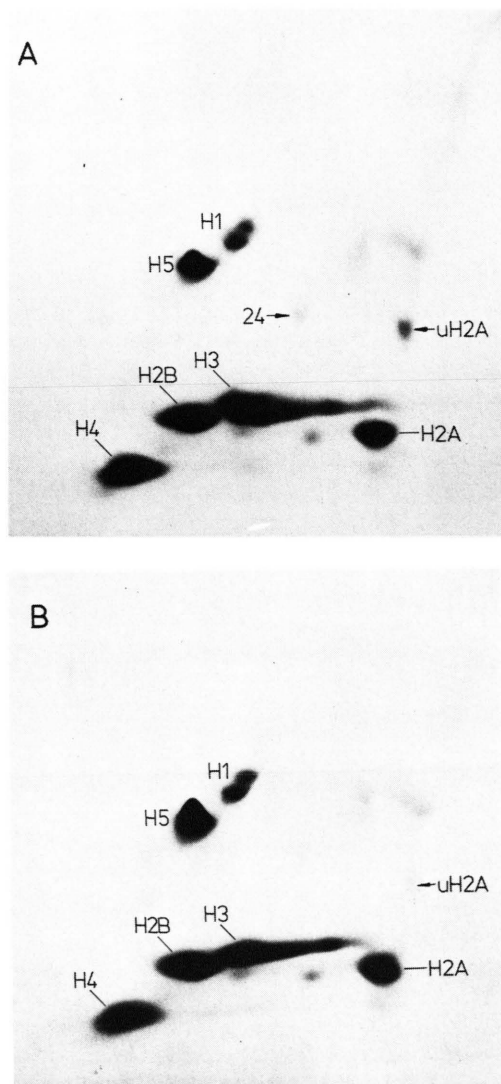

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of chromatin acid-soluble proteins from hen reticulocytes (A) and erythrocytes (B). Approximately 300 μg of protein was separated as described in Fig. 1.
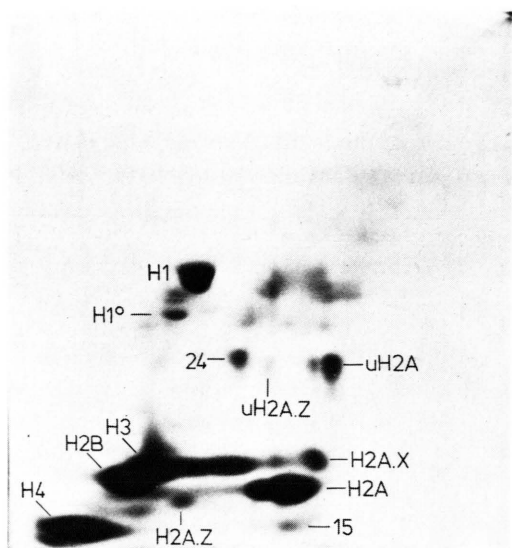
Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of acid-soluble chromatin proteins from rabbit liver. Proteins were isolated from nuclei washed with 0.14 and 0.35 M NaCl solutions and separated in the first dimension in 8% acetic acid-urea polyacrylamide gel containing 0.36% Triton X-100 and in sodium dodecyl sulfate slab gel in the second dimension.

In Fig. 2 patterns of histones extracted from reticulocyte and erythrocyte chromatin are shown. In general, the amounts of reticulocyte H2A histone variants were much lower in relation to main histone band in comparison with those of hen liver but 24-kDa and uH2A proteins were still well visible. However, 24-kDa protein was completely absent and uH2A was only barely detectable in erythrocyte chromatin. This observation further strongly supports the assumed dependence of 24-kDa protein upon extent of RNA synthesis in nuclei because erythrocytes are known to possess a little RNA in chromatin [41] due to their inactivity in transcription [29].

Discussion

D-galactosamine is considered to be a powerful agent causing a serve disturbances in RNA pool of liver cells [27, 28] by inhibiting its synthesis by more than 90% within 0.5−2 h after injection. It has been demonstrated [28] that both transcription of nucleolar and nucleoplasmic genes in rat liver was inhibited. This led to a lowering of labelling of nuclear proteins within 2 h [28] and a sharp decrease of chromosomal RNA and nonhistone proteins content

6 h after administration of this compound [42]. In addition, both quantitative and qualitative differences were detected in the gel patterns of total and residual chromatin proteins from liver of galactosamine-treated rats [42, 43] demonstrating a relationship between existence of some nuclear proteins and continuous RNA synthesis in the cell.

Results presented in Table I revealed changes in the amount of chromatin proteins in hen liver after D-galactosamine injection. It appears that alteration of protein content in hen liver chromatin after galactosamine treatment may be attributed to the changes in chromatin nonhistone proteins as it was shown earlier by Weiss et al. [42]. Electrophoretic analysis of histones isolated from liver of normal and galactosamine-treated birds revealed a striking similarity of their spotting patterns. Only a protein with molecular weight of approximately 24 kDa was shown to decrease in comparison with normal hens (Fig. 1). Surprisingly, previous studies conducted in this laboratory [30, 44] have also revealed only a limited alterations in two-dimensional polyacrylamide gel patterns of nucleoplasmic and nonhistone chromatin proteins from chick and hen liver following D-galactosamine administration.

The lower amount of 24-kDa protein spot in gel patterns of acid-soluble proteins from galactosamine-treated hens was correlated with a decrease of total RNA and protein content in liver chromatin of those birds. This observation seems to indicate that the level of this protein might be closely related to the level of RNA synthesis and/or structural as well as functional rearrangements of the liver chromatin as a result of galactosamine injection. This explanation was further supported by electrophoretic analysis of acid-soluble proteins from chromatin of mature erythrocytes (Fig. 2) which are inactive in RNA transcription [29]. The 24-kDa acid-soluble protein present in a detectable amount in the reticulocyte chromatin disappeared completely from gel patterns of histones extracted from hen erythrocyte chromatin. It is worth noting that the amount of other protein, the uH2A (protein A24 of Goldknopf et al. [17]), was much lower in erythrocytes in comparison with reticulocytes. This observation is in perfect agreement with data of Goldknopf et al. [17] who pointed out that amount of uH2A protein declines simultaneously with decrease of RNA transcription during erythropoiesis. They have suggested that the presence of uH2A protein in premature erythrocytes

may reflect the presence of potentially active and transcribing chromatin structures.

It appears that 24-kDa protein represents a unique nonhistone protein coextracted with histones. Electrophoretic mobility and localization of this protein on the two-dimensional slab gel differ from those of H2A histone variants and their adducts with ubiquitin. The nearest ubiquitinated protein, the uH2A.Z, was found to be placed between the 24-kDa and uH2A proteins as it was shown in gel patterns of acid-soluble proteins extracted from rabbit liver chromatin (Fig. 3) and hen liver nuclei [45]. The molecular weight and electrophoretic properties of 24-kDa protein differ also from those of acid-soluble nonhistone proteins described previously by others [22, 24–26].

In this work the effect of D-galactosamine on gel patterns of histones and other acid-soluble proteins of liver chromatin was compared not only in normal but also in starving hens. The status of fasting differs significantly from normal one because it produces a hypometabolic conditions for biochemical reactions. Starvation induces also hypothyroidism by dropping a serum thyroxine and triiodothyronine levels and reducing liver nuclear triiodothyronine receptors [46]. Recently, a decrease of nuclear protein referred to as a n-band with molecular weight of about 125 kDa and an increase of a t-band with molecular weight of about 80 kDa have been observed in the liver of starved rats [3, 4]. The n-protein may be partially restored by giving a physiological dose of triiodothyronine, 2 μg of T_3 daily/100 g body weight, for a week. This was not in the case of the t-band indicating that it was strongly depended on nutritional status of animals [4]. Results presented in this work (Fig. 1) show that except for 15- and 32-kDa proteins there were no significant changes in the two-dimensional gel pattern of acid-soluble chromatin proteins from fasted chickens. Thus, prolonged fasting failed to induce the alterations of main spots of basic chromatin proteins. Recently, Luboń and his coworkers [45] have reported that only a slight lowering of a 27-kDa protein migrating just ahead of H1 histone took place following transitory exposure of hens to starvation. It is possible that observed diminution of protein content in chromatin of starved hens (Table I) did not result from a lowering of histone/DNA ratio but was rather due to a decrease of the amount of nonhistone proteins. It is well known [5] that histones play a very significant role in the

structure and function of chromatin so that their removing would produce a profound disturbances in chromatin. This assumption is supported by observations which indicate that – in contrast to nonhistone proteins – the amount of histones in animals fed a protein- or essential amino acid-restricted diet is almost unchanged [47–50].

Fasting is known factor influencing the susceptibility of animals to hepatotoxic injury [51]. In particular it reverses the liver cell response to the galactosamine treatment because there was significantly higher liver glycogen content in starved rats injected with D-galactosamine in comparison with normal ones [52]. Although starvation did not change the amount of 24-kDa protein (Fig. 1B), this protein was only barely detectable in patterns of acid-soluble proteins from fasting birds receiving D-galactosamine. As evidenced in Fig. 1C, the galactosamine injected to normal hens caused also a decrease of the amount of this protein but, however, to a lower degree. Thus, the disappearance of this protein in hen liver chromatin after D-galactosamine administration is assumed not to depend only on the inhibition of RNA synthesis because it was surprisingly still present in constant amount in starved hens having a reduced content of chromatin RNA (Table I). Presumably, the diminution of this protein could be also connected with other factors which might be activated following galactosamine administration to hens or might appear during reticulocyte differentiation.

The alterations of 24-kDa protein levels in transcriptionally inhibited cells (Fig. 1D and Fig. 2) closely resemble those of protein uH2A described by Goldknopf et al. [17, 53] during erythrocyte maturation. They have found that transcriptional shutdown during final maturation of the chicken erythrocyte was accompanied by losses of nuclear uH2A protein, ubiquitin portion of uH2A protein and endogenous uH2A protein lyase activity. It seems that a selective loss of the 24-kDa protein following D-galactosamine administration to normal and starving hens might be explained by activation of a specific protease which could preferentially cleave it. The induction of such enzyme which specifically degraded conjugate protein uH2A was reported to occur in liver nucleoli from thioacetamide-treated rats [54]. It can not be excluded, however, that 24-kDa proteins may turn over so rapidly within chromatin that its disappearance after D-galactosamine injection could be simply caused by a lack of specific mRNA which in turn

precludes its continuous synthesis. This explanation appears to be valid because the remaining major and minor protein spots that failed to undergo substantial alterations could represent a relatively stable chromosomal proteins.

Further studies are needed to test whether the loss of 24-kDa protein from hen liver chromatin following D-galactosamine administration and during chicken erythrocyte maturation represent a unique event accompanying the chromatin inactivation as described earlier [17] for uH2A protein during chicken erythrocyte differentiation.

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