Zonal Electrophoresis of the Soluble Nuclear Proteins of Normal and Preneoplastic Livers

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SUMMARY

The saline-soluble proteins of rat liver nuclei have been extensively resolved into profiles according to charge, permitting comparison with similar arrays from liver cytoplasm.

Nuclei were isolated in 2.1 M sucrose solution from the perfused livers of rats fed stock, control, or hepatocarcinogenic 3'-methyl-4-dimethylaminoazobenzene diet. After feeding the azo dye for 20 days, the nuclei contained 3% of the bound azo dyes of liver. Exhaustive extraction of the nuclei in isotonic saline-phosphate buffer at pH 7.4 dissolved approximately 49% of the nitrogen and 86% of the protein-bound dyes.

Fifteen classes of the soluble nuclear macromolecules, formerly designated on the basis of free boundary electrophoresis, were isolated by column zonal electrophoresis. The strongly acidic, weakly acidic, and near-neutral Classes 2—15 consist of proteins. In contrast, the basic Classes 16—18 are composed of small molecules, apparently dissociated from macromolecules. Approximately one-half of the protein-bound dyes in the charge profiles are associated with a small class of near-neutral proteins, Class 14. The remainder are combined mainly with the acidic proteins, Classes 5—9.

The saline-soluble proteins isolated from liver nuclei generally appear to be similar to those of cytoplasm. This appears to apply particularly to the principal near-neutral azoproteins of the nucleus (Class 14) and those of the cytoplasm (slow h2). Conjecturally, these soluble macromolecules may be shared throughout the "soluble space" of the cell.

INTRODUCTION

Numerous investigations have sought to characterize the components and functions of the cell nucleus. However, relatively few studies have focused on the saline-soluble macromolecules which presumably originate from the nuclear sap (compiled (3)). Recently, these macromolecules of rat liver were analyzed, revealing a great complexity of many size and charge classes. The classes were identified and their relative amounts determined (3). Further characterization requires that these classes be isolated. Such fractionations have been developed for the liver cytoplasmic soluble proteins (29, 33, 34). If procedural conditions were similar, comparisons of both subcellular systems would also be possible.

Metabolites of chemical carcinogens combine in vivo least with DNA, more with RNA, and most with certain proteins of the target organs [reviewed (19, 21)]. One or more of these interactions may be critical in the carcinogenic process.

Some carcinogens exhibit target specificity in vivo in their combinations with tissue proteins. Thus, protein conjugates of the aminoazo dyes, N-2-fluorenylacrylamide (2-acetylaminofluorene), and polycyclic hydrocarbons occur mostly in cytoplasmic sap (15, 18, 22, 37) belonging primarily to a small electrophoretic class of near-neutral proteins, the h2 (1, 6, 8, 28, 31). Various correlations appear to implicate the h2 conjugates in the carcinogeneses induced by these three types of carcinogens (1, 3, 6, 8, 28—31, 34).

With respect to the aminoazo dyes, the presence of small amounts of protein conjugates in cell nuclei (9, 10, 22, 24) nevertheless permits the possibility that the critical carcinogen interaction may be in this compartment. Gross fractionation of the nuclear proteins suggests a degree of target selectivity by the azocarcinogens (9, 24). For example, azo dye adducts of histones have not been detected (7, 9). In contrast, the saline-soluble nuclear proteins contain bound azo dyes (3, 4) and bound metabolites (24) derived from the azocarcinogens.

Therefore, the present study (a) presents a procedure for the preparative isolation of the individual charge classes of the saline-soluble macromolecules of liver nuclei, (b) characterizes the heterogeneity of the azoproteins therein after azocarcinogen feeding, and (c) compares the nuclear and cytoplasmic systems in these regards.

MATERIALS AND METHODS

Rats and Diets. Adult male rats (375—425 gm) of CFN strain (Carworth Farms, Inc.) were fed the same diets as in the previous study (3). Normal rats were given stock pellets (Wayne Lab Blox). Control and experimental rats were fed for 20 days synthetic diets lacking and containing 3'-methyl-4-dimeth-
ylaminoazobenzene (3'-Me-DAB) respectively. The rats were fasted 17 hr prior to sacrifice.

**Solutions.** All solutions were prepared in double-distilled water. Saline-phosphate buffer consisted of 0.12 M NaCl + 0.01 M sodium phosphate buffer, pH 7.4. Veronal-Cl-Mg buffer contained 0.03 M NaCl, 0.5 mM MgCl₂, and 0.02 ionic strength sodium veronal buffer, pH 8.6.

**Isolation of Soluble Nuclear Proteins.** Except as noted, all procedures were carried out in the cold as formerly described (3). Briefly, perfused livers were homogenized in 2.1 M sucrose with a rotating pestle. The suspension was screened, diluted with sucrose solution, and centrifuged at 40,000 × g (average) for 60 min. The nuclei thus isolated were extracted 4 times with 0.12 M NaCl + 0.01 M NaPO₄, pH 7.4, in a pestle homogenizer with intervening centrifugations at 20,000 × g (average) for 10 min. The soluble nuclear macromolecules were isolated by filtration through Sephadex G-25 gel into veronal-Cl-Mg buffer, then concentrated by dialysis against this buffer containing purified dextran, and dialyzed against the buffer without dextran. The protein concentration was adjusted to approximately 6% for column electrophoresis.

**Column Electrophoresis.** Zonal electrophoresis was carried out at 2.2°C in columns of 220 × 3.1 cm, i.e., of nitrosylated ethanolized cellulose as previously described (29, 34). In order to minimize the loss of acidic constituents on the cellulose stabilizer (see below), columns were operated in veronal-Cl-Mg buffer. The presence of Mg²⁺ in the veronal-chloride buffer used previously (3, 34, 35) appeared to have had little effect on the protein profile.

An 8-ml aliquot of the protein solution was entered into the column to a level 2 mm below the cellulose surface. The protein zone was covered with a 4-cm layer of cellulose added in deaerated cold buffer. Electrophoresis was then carried out for 122 hr using 80 ma in the column circuit (120—125 ma total).

**Preparation of Nitrosylated Cellulose.** One kg of ethanolized “superregenerated” cellulose (29) was suspended in 16 liters of 0.1 M HCl containing 0.1 M NaNO₂. After mixing for 45 min by polyethylene stirrer, the cellulose was filtered rapidly on a Büchner funnel. The cake was immediately washed twice by polyethylene stirrer, the cellulose was filtered rapidly on a Büchner funnel. The cake was immediately washed twice by filtration through Sephadex G-25 gel into veronal-Cl-Mg buffer, then concentrated by dialysis against this buffer containing purified dextran, and dialyzed against the buffer without dextran. The protein concentration was adjusted to approximately 6% for column electrophoresis.

**Assays.** Protein content was measured spectrophotometrically by 284 μAₙ (A₂₈₄) or by biuret reaction standardized with human serum.

Total (weakly and firmly) bound azo dyes were assayed by a modification of the formic acid method (14). Aliquots of the solutions applied to the electrophoresis column and of eluted column fractions were mixed with 0.5 ml of veronal-Cl-Mg buffer containing 10 μg pancreatic ribonuclease (Armour Co., Chicago, Ill.) and incubated for 30 min at 40°C in a shaking bath. Each digest was mixed with 0.5 ml of veronal-Cl-Mg buffer containing 40 μg pronase³ (Calbiochem., Los Angeles, Cal.), and shaken in a 40°C bath for 16—18 hr. In order to test the completeness of protein digestion, samples with the most protein (A₂₈₄) were heated for 2 min at 100°C. If they developed turbidity, more pronase was added to every tube and digestion was continued for an additional 4 hr. The digests were then transferred into 25 × 200 mm heavy wall test tubes, shell-frozen, covered with bolting silk (60 mesh), and freeze-dried. Each powder was dissolved in 0.5 ml of 88% formic acid, and read in 1-cm micro cells at 400 μm and 525 μm in a Beckman DU spectrophotometer. Reference blanks contained the same amounts of lyophilized buffer and of enzymes. Because most fractions contained interfering pigments which absorbed moderately at 525 μm in formic acid, the dye absorbancies at 525 μm (A₅₂₅) in dye profiles were corrected using absorbancies at 400 μm (A₄₀₀) and 525 μm in control profiles and at 400 μm in dye profiles. The correction was made according to the formula employed in the studies on cytoplasmic extracts (34). For substantiation of the presence of azo dyes, absorption spectra of selected fractions and of unfractionated nuclear extracts were obtained in a Beckman DK-2 recording spectrophotometer.

Firmly bound azo dyes were assayed by the extraction-digestion method of Miller and Miller (17, 20).

Lactic dehydrogenase (LDH) was assayed spectrophotometrically as described by Weber and Cantero (36).

**RESULTS**

**Recovers.** Based on assay of DNA, the recovery of nuclei from the filtered homogenate was 71%. As in the previous study (3), microscopic examination under oil immersion showed the isolated nuclei to be fairly well preserved and essentially free of visible cytoplasmic contamination.

Extraction of nuclei with saline-phosphate buffer solubilized 49 ± 2% of the nuclear nitrogen. Approximately 8% of the absorbance at 284 μm (A₂₈₄ mμ) of the unfiltered 22,000 × g supernatant extract was retained by the G-25 Sephadex gel during removal of small molecules. An additional 2—4% was discarded, since it was present in the gel elution profile as a highly dilute shoulder on the macromolecular component. The concentrated pool applied to the electrophoretic column contained 85 ± 3% of the protein (A₂₈₄ mμ) extracted from the nuclei.

When nuclear proteins were resolved by zonal electrophoresis in 0.02 ionic strength veronal buffer, pH 8.6, containing 0.03 M NaCl, the buffer used previously for free boundary electrophoresis (3), protein yields in the eluates were 65—68% of the protein added to the columns. Addition of 0.5 mM MgCl₂ to this buffer improved the recoveries, and in 17 experiments the corresponding yields averaged 84 ± 7%. In 4 experiments, including 3 controls, recoveries were below 75%. All 4 were rejected. The most loss was apparently suffered by the weakly acidic Components 5 and 6, and less by Components 3 and 4 (see below). An additional 3—5% of the applied sample could have been recovered, if the polyamionic and highly acidic Components 1 and 2 were collected in a dialysis bag affixed to the bottom of the column (34).

Firmly bound azo dyes were found to be present in liver nuclei after ingestion of 3'-Me-DAB, in confirmation of previous reports (22). The accompanying paper (5) reports that on the basis that nuclei comprise approximately 7% of the liver cellular matter (25), the sucrose-isolated nuclei contained

**Soluble Proteins of Liver Nuclei**
Protein Profiles. The column electrophoretic profiles of the soluble nuclear proteins from rats fed the 3 diets were generally similar. This is shown in Chart 1, which contains representative patterns of the 3 normal, 2 control, and 5 dye experiments. The profiles resembled the patterns obtained previously by free boundary electrophoresis under similar conditions (3). The formerly used nomenclature of components was therefore adopted in this study. Certain reference markers assisted in the designations of components. In liver cytoplasmic extract, the

![Chart 1](image-url)

Chart 1. Zonal electrophoretic profiles of the soluble proteins of aqueous-isolated nuclei of the livers of rats fed stock, control, and 3'-methyl-4-dimethylaminobenzene (3'-Me-DAB) diets. Levels of lactic dehydrogenase (LDH) are plotted as ml x O.D./min. Protein recoveries in the column eluates were 86%, 85%, and 98% in the top to bottom profiles respectively.
principal isozyme of lactic dehydrogenase, LDH-V, belongs to middle $h_2$ component, and the main azoprotein to the slow $h_2$. Since they have the same electrophoretic mobilities as have the nuclear Components 13 and 14 respectively (3, 34, 35), the LDH-V and dye peaks in nuclear profiles established the locations of Components 13 and 14 in the profile (Chart 1). Components 11 and 17 were usually identified by their relative discreteness. All others were assigned to positions proportionate to their free boundary electrophoretic mobilities (3). The validity of the treatment was supported by the agreements of the relative areas and resolutions of almost all components in both types of electrophoresis. The exception was the Component 18. It was retarded and tailed extensively, reflecting its relative great basicity (Chart 1).

According to biuret reaction, Components 2 through 15 contained protein, while Components 16, 17, and 18 did not (Chart 1). The constituents of the latter components also did not react with ninhydrin nor precipitate in 10% trichloroacetic acid, and were dialyzable. This indicated presence of free small molecules is surprising, since only macromolecules had been prepared for electrophoresis (see Discussion).

In the 3 experiments with nuclear extracts of normal liver, the relative amounts of the highly acidic, weakly acidic, and near-neutral Components 2 through 12 deviated only slightly. In contrast, Components 13 through 18 varied, particularly so Components 15 through 17.

Azoprotein Profiles. Feeding the azocarcinogen for 20 days results in the appearance of specific soluble nuclear azoproteins in liver. These belong mainly to the near-neutral Component 14. This is shown by the shaded profile in Chart 1. The specific concentration ($A_{525}/A_{284}$) of bound azo dyes in this region is at least four-fold greater than at any other component (2 at Component 14 compared to 0.2, 0.1, 0.5, 0.4 at Components 5, 6, 8, 9 respectively). Moreover, since the peak of the azo dyes at Component 14 falls between two large protein peaks, its specific concentration after further resolution would undoubtedly be considerably greater than 2. The nuclear azoproteins more acidic (anionic) than those of Component 13 were not only of lower specific concentrations, but of greater heterogeneity. Further resolution would probably show that their specific concentrations are actually very much smaller than that of the azoproteins of Component 14.

The nuclear azoproteins of Component 14 are bordered in the profile by minor amounts of azoprotein of the near-neutral Components 13 and 15. There is a shoulder on the main peak at Component 15 and a slight skewing at Component 13. In the absence of further resolution, the relative amounts in these two regions cannot be accurately assessed. The azoproteins in the Components 13 through 15 account for 53% of all the bound dyes in the nuclear extract. Those at Component 14 constitute the most by far, estimated at 48%. These principal nuclear azoproteins of Component 14 are equivalent to the principal soluble cytoplasmic slow $h_2$ azoproteins, both migrating with the same electrophoretic mobility (3, 34, 35).

A graphical illustration of the heterogeneity of the azoproteins of Components 13 through 15 can be seen in Chart 2. Throughout the span of Components 11–16, levels of total azo dyes and LDH isozyme V were normalized as percentages of their peak values vs elution volumes. Both plots were obtained under matching column conditions. According to this analysis, the azoproteins in the main peak are considerably more heterogeneous than is the LDH isozyme. Their width at half height is approximately three-fold greater than that of the LDH. A similar comparison has been made for the cytoplasmic slow $h_2$ azoproteins (29, 34). By this test a number of supposedly "single" cytoplasmic enzymes are considerably more heterogeneous than is cytoplasmic isozyme LDH-V (29).

Nuclear extracts of control and 3'-Me-DAB livers contain chromogens, as do the analogous extracts of cytoplasm (34). The chromogens interfered in the assay of total protein-bound azo dyes and necessitated the correction described under Methods. The shaded profile in Chart 1 represents bound azo dyes after such correction. The interfering constituents including hemes are shown in Chart 1 to absorb at 400 mμ in the control and 3'-Me-DAB profiles and at 525 mμ in the control.
profile. They are present mainly among the weakly acidic proteins of Components 5 through 9 and the near-neutral proteins of Component 10. In this span, absorbance at 400 μM is at least twice that at 525 μM.

DISCUSSION

The saline-soluble proteins of liver nuclei have been resolved into profiles according to charge, providing an array of proteins in sizeable amounts suitable for numerous studies otherwise unfeasible. This investigation is one of a number dealing with the saline-soluble macromolecules of nucleus and cytoplasm of rat liver (5, 29, 33—35). Coupling information gained from both subcellular systems provides greater perspective than would otherwise be the case. That an electrophoretic fractionation of the saline-soluble proteins of liver nuclei has been developed as part of this comparative scheme is a principal outcome of this study.

Despite its advantages as an anticonvection stabilizer for zonal electrophoresis, ethanolized cellulose is not ideal. In some cases it interacts with macromolecules, resulting in irreversible absorption, dissociation, and chromatographic retardation. To reduce these effects, the ethanolized cellulose of Flodin and Kupke (11) was subjected to alkali-acid washes (29). This product has worked well for the soluble cytoplasmic proteins (29, 34), but not for the nuclear proteins. One source of the trouble is that the material contains a slight amount of alkaline groups (5—6 microequivalents per gm). These have been reduced by treatment with nitrous acid. The product constituted an improvement, but still some nuclear acidic proteins were lost. This was overcome in part by the addition of MgCl₂ to the standard veronal-Cl buffer. Presumably the Mg²⁺ combined with the phosphate groups of ribonucleoproteins, thereby reducing their interaction with the cellulose.

Zonal electrophoresis has made feasible the characterization of the many types of soluble proteins of liver nuclei. Thus, all but the basic Components 16, 17, and 18 consist of macromolecules. The small molecular size and high basicity of the constituents of Components 16, 17, and 18 suggest that they may possibly consist of ultraviolet-absorbing oligoamines, analogous to but not spermine and spermidine (23). The appearance of small molecules in the column profile of macromolecules previously isolated by gel filtration indicates that dissociation of salt-linked constituents occurs during the electrophoretic process. The cause is not related to the cellulose. per se, since Components 16, 17, and 18 also occur in free boundary electrophoresis (3).

The significance attributable to findings regarding the soluble proteins of liver nuclei has previously been questioned (3). Uncertainties concerning the biochemical purity of the nuclei, the exclusiveness and totality of the extraction of the nuclear sap, the nativeness of the solubilized macromolecules, and the extent of cytoplasmic contamination all require that conclusions be qualified. The concern is supported by the reported penetrability of various substances including macromolecules into isolated nuclei (2, 16). Thus, most of the nuclear soluble azoproteins conceivably may be cytoplasmic contaminants incorporated during the isolation of nuclei, particularly since they contain only 3% of all the protein-bound dyes of liver.

That the dye concentration per mg protein in nuclear extract is comparable to that in liver may weigh for or against this possibility (see directly below). In the hope of arriving at a firmer evaluation of the composition of the soluble macromolecules of liver nuclei in vivo, aqueous and nonaqueous nuclei have been compared in a parallel study (5).

Most of the soluble macromolecules of nuclei and cytoplasm may be shared in common throughout the "soluble space" of the cell. This is in agreement with our previous view (3) and the thesis of Siebert (reviewed (27)). Both subcellular systems are analogous in the following ways: (a) Their electrophoretic profiles are quantitatively and qualitatively similar. This is shown in Chart 6 of the previous report (3). The mobilities of the nuclear classes also match those of the cytoplasmic extract. This applies in particular to the five near neutral nuclear Components 10—15 and the five cytoplasmic h components. Both extracts are now known to have nearly identical limits of mobility of their most basic macromolecules (Component 15 and h₂) (this report and (3, 34, 35)]. (b) An analogous species of LDH isozyme appears to be present in both extracts. This is in agreement with the prior finding of Siebert and Hanover (26). (c) The principal species of azoproteins from nuclei and cytoplasm are similar, if not identical. As part of the nuclear Component 14 and cytoplasmic slow h₂ component, both have the same mobility [(3); this report]. Further, their protein-bound dyes appear to be present in nuclei and cytoplasm at similar relative concentrations. This is based on the estimation that the amount of all bound dyes per mg protein in aqueous nuclei is about 43% of that in cytoplasm (5). Approximately 86% of the nuclear bound dyes are soluble (this report), and likewise about one-half in cytoplasm (22). Each of the two species of specific azoproteins contains about one-half of the protein-boundazo dyes in its extract (this report and (28, 34)]. It follows that the relative content of protein-bound dyes per mg in Component 14 of nuclei is approximately three-fourths of that of the slow h₂ of cytoplasm (0.43 x 0.86 x 0.50/1.00 x 0.50 x 0.50).

The search for the essential interactions between activated carcinogens and tissue macromolecules is currently a key problem in the elucidation of possible instructional mechanisms of chemical carcinogenesis. Nucleic acids, particularly DNA, and certain proteins are at present primary candidates for the role of the critical target in the neoplastic transformation. The proteins most likely to be involved as products of the interactions between target proteins and activated carcinogens are certain of the h₂ proteins (Introduction). The h₂ proteins of normal rat liver were recently found to inhibit the multiplication of various cells in vitro (12). This resulted from the depletion of arginine in the nutrient medium. The effect is due to the localization of liver arginase in the same slow h₂ fraction which in dye-fed rats contains the principal soluble azoprotein (13, 32). It remains for future study to determine whether this and other circumstantial evidence apparently relating the h₂ azoprotein and arginase is fortuitous or is relevant to azo dye liver carcinogenesis (32).

The present findings suggest that the h₂-like conjugates of Component 14 constitute the principal species of azoproteins in liver nuclei. Accordingly, the h₂ conjugates and their normal unconjugated target proteins may occupy the con-
jected "soluble space" of the cell, permeating both cytoplasm and nucleus. With continued carcinogen conjugation, interchange may conceivably result in depletion of the normal protein target from its site of action either in cytoplasm or in nucleus.

ACKNOWLEDGMENTS

The able technical assistance of Mr. Walter Tkaczcyk and Miss Claire McKeever is gratefully acknowledged.

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